DNA Analysis of Ornithine Transcarbamylase (OTC) Deficiency in South African patients

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A thesis submitted to the Faculty of Medicine / Health Sciences, University of Cape Town in fulfilment of the requirements for the degree of Master of Science

Abstract

Hyperammonaemia is not an infrequent presentation in the newborn or neonatal period. While the majority are transitory in nature and due to infective processes or liver pathology/immaturity, a significant number are due to defects in enzymes of the urea cycle. This cycle has evolved to cope with waste nitrogen disposal and the de novo synthesis of arginine. There are five distinct enzymatic steps in the urea cycle, and defects in each, result in a biochemically distinct disease. Four of these diseases, deficiencies of carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinic acid synthetase (ASS), and argininosuccinate lyase (ASL) can present dramatically within the first 24 to 48 hrs of life with progressive lethargy, hypothermia and apnea, all related to very high plasma ammonia levels. These diseases may also present later in infancy, childhood and adulthood with hyperammonemia and episodic mental status changes. The fifth defect, arginase deficiency presents as progressive spastic quadriplegia and mental retardation but with milder elevation of blood ammonia levels. The molecular genetics of these disorders in South Africans has not been explored and there is thus very little information on phenotype/genotype relationships, specific for citizens of this country. This study aims to correct this imbalance and has concentrated initially on OTC deficiency, which is X-linked and therefore the most common defect encountered. Initial work on this project has concentrated on subjects with a classical X-linked OTC phenotype.

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Abbreviations and Symbols

α	alpha
β	beta
µl	microlitre
µM	micromolar
A	adenine
A_{260}	absorbance at 260nm
A_{280}	absorbance at 280nm
AD	autosomal dominant
AR	autosomal recessive
A	arginase
ASL	argininosuccinate lyase
ASS	argininosuccinate synthetase
AMP	adenosine monophosphate
APS	ammonium persulfate
ATC	aspartate transcarbamylase
ATP	adenosine triphosphate
bp	Base pairs
C	cytosine
C-terminal	carboxy terminal
cDNA	complimentary DNA
cM	centiMorgan
COOH	carboxy terminal
CP	Carbamyl Phosphate

CPS	Carbamyl Phosphate synthetase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddTTP	dideoxythymidine triphosphate
ddH_2O	deionized distilled water
dH_2O	distilled water
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
G	guanine
G6PD	Glucose-6-phosphate dehydrogenase
ICH	Institute of Child Health
IEM	inborn errors of metabolism
IMD's	Inherited metabolic disease
kb	Kilobases
L	liter
M	Molar
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MW	molecular weight
N-terminal	amino terminal
NH_3	nitrogen

nm	nannometre
OD	optical density
OTCD	Ornithine Transcarbamylase Deficiency
PAGE	polyacrylamine gel electrophoresis
PCR	Polymerase Chain Reaction
RNA	ribonucleic acid
RDT	recombinant DNA technology
RT	room temperature
RXH	Red Cross Children's Hospital
SSCP	single stranded conformational polymorphism
T	thymine
Temed	N,N, N',N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UV	ultraviolet

Declaration

I, Liezel Catharine Swarts, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

May 2004

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After all those years, I've got quite a list of people who contributed in some way to this thesis, for which I would like to express thanks.

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CHAPTER 1

1. Introduction

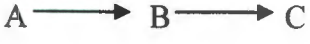
1.1 Inherited metabolic diseases

This study deals with deficiencies of one of the enzymes of the urea cycle, and which fall into the category of inherited metabolic diseases (IMDs), sometimes referred to as inborn errors of metabolism (IEMs). These are largely monogenic disorders in that they are caused by the absence or dysfunction of a single gene product. As a group, they differ significantly from the chromosomal disorders where entire chromosomes or sections of chromosomes can be duplicated or lost from the human genome. In these instances, many contiguous genes can be duplicated or removed, sometimes resulting in unusual and complex metabolic derangements. (Harley E, 2000)

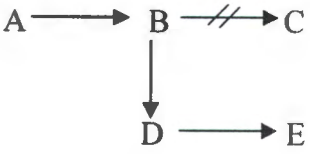
The number of genes coding for enzymes in the human genome must number in the thousands, which raises the theoretically possibility of a corresponding number of IMDs. Reviews attempting to deal with just the portion of known enzymes deficiencies, have run to several volumes as typified by the 4 volume set "The Metabolic and Molecular Bases of Inherited Disease" (Scriver, C.R. *et al* 2000). Given this context, the best compromise for this thesis is to record the general principles of the genetics, diagnosis, and treatment. (URL. 1)

1.1.1 General concepts

A typical metabolic pathway might show substrate A being converted to product, C through an intermediate B:



Should there be a block in the conversion of B to C, then an alternative pathway may be activated with B being metabolized to products D and E:



The metabolic consequences of an enzymatic block in the conversion of B to C, may be due to excess of precursors (A and B), the absence of product (C), or excessive amounts of alternate products (D and E). Their impact will vary between individuals as the degree of enzyme loss, kinetics of alternate pathways, and endogenous production of precursors differs between patients. The phenotype for a given disorder can thus be quite variable. Both exogenous and endogenous factors, which cause an increased metabolic flux through the defective pathway, can result in metabolic crisis and therefore, clinical deterioration. Endogenous catabolism generates the bulk of metabolites and becomes prominent in catabolic episodes induced by illness, fever, and starvation. These situations thus often precipitate or aggravate IMDs. (URL. 1)

1.1.2 Classification of IMDs

IMDs have many classification schemes, with one of the most prominent dividing the disorders into categories of cellular intoxication, energy deficiency, and of mixed types. Cellular intoxication occurs in disorders such as those involving the urea cycle enzymes or amino acid metabolism, and lysosomal function. In these cases, cells are poisoned by either the accumulation of small molecules which inhibit other metabolic pathways, lower intracellular pH etc, or by the accumulation of large molecules such as lipids or polysaccharides, which precipitate in the cytoplasm. Energy deficient disorders, such as pyruvate dehydrogenase deficiency (PDH) or medium chain acyl-CoA dehydrogenation deficiency (MCAD) exert their effects by depriving cells of the energy they need to function properly; this occurs through deprivation of substrates for the citric acid cycle. The mixed category is self explanatory and includes disorders such as the peroxisomal defects. (URL. 1)

1.1.3 Genetics

Most IMDs are autosomal recessive, occurring when an individual inherits two alleles with defects at a specific locus. This occurs infrequently in the general population but at a heightened frequency in unions between related individuals ie in consanguineous marriages. Others are inherited as X-linked recessive and

are more prominent as only one defective allele leads to disease in males. Examples include haemophilia, deficiencies in Glucose-6-phosphate dehydrogenase (G6PD) (causes a haemolytic anaemia), Duchenne muscle dystrophy and OTC (ornithine transcarbamylase), which is the focus of this thesis.

In X-linked recessive disorders, hemizygous males are almost always symptomatic whereas carrier females may or may not present with clinical symptoms depending on their lyonization patterns. Carrier females have a 50% risk of passing the gene onto their children. Theoretically then, half of the boys could be affected while half the daughters could be carriers.

1.1.4 Genes and IMDs

Recently, recombinant DNA technology (RDT) has shown new ways to understand, diagnose and treat inherited diseases. RDT has relevance to IMDs in three ways; firstly, both normal and mutant genes can be cloned and studies; secondly, the technology provides novel and powerful diagnostic procedures and thirdly, treatment, through gene therapy, is now available to replace or insert copies of a normal gene to compensate for the defective gene. (Harley E, 2000)

1.2 The urea cycle

1.2.1 Overview

Urea is the chief nitrogenous excretion product of mammals. It is largely synthesized in the liver and involves a series of 5 reactions that are distributed between the mitochondria and the cytosol. This series of reactions is known as the Urea Cycle or the Krebs-Hensleleit Cycle. (URL. 2)

Most of the nitrogenous waste comes from the breakdown of amino acids and this occurs by deamination, which results in the production of ammonia (NH_3). Ammonia is very toxic and its continued accumulation in the body is fatal. However, the liver contains a system of carrier molecules and enzymes that quickly

convert the ammonia into urea for excretion by the kidneys. A second function of the urea cycle is the *de novo* synthesis of arginine. (Brusilow and Horwich, 1995)

High protein diets are particularly demanding of the urea cycle as the high nitrogen excess must be excreted. This is achieved by up regulation of the genes of the urea cycle. If these diets persist then 20-fold or greater increases in enzymes concentrations can be encountered. Elevated cycle enzymes are also found in situations of starvation when muscle breakdown accelerates. (URL. 2).

1.2.2 Biochemistry

The essential features of the urea cycle reactions are depicted in figure 1 and can be described as follows: Carbamyl Phosphate synthetase (CPS), an enzyme from the mitochondrial matrix, catalyzes the biosynthesis of Carbamyl Phosphate (CP) from ammonia and bicarbonate. Ornithine which is found in the cytosol is then transported to the mitochondrial matrix where it is condensed with CP to produce citrulline. This reaction is catalyzed by OTC and the energy for this reaction to take place is provided by the high-energy anhydride of CP. Citrulline is the transported to the cytosol, where it is condensed with aspartate to form argininosuccinate. This reaction is catalyzed by argininosuccinate synthetase (ASS). The cytosolic enzyme argininosuccinate lyase (ASL), leads to the production of arginine and fumarate. In the final step of the cycle, arginase cleaves urea from arginine, generating ornithine. Ornithine is then transported back to the mitochondrial matrix for another round of urea synthesis. A total of 3 ATPs and phosphates are consumed during the reaction of this cycle and the only new compound generated by this cycle is urea (Fig. 1). (URL 2)

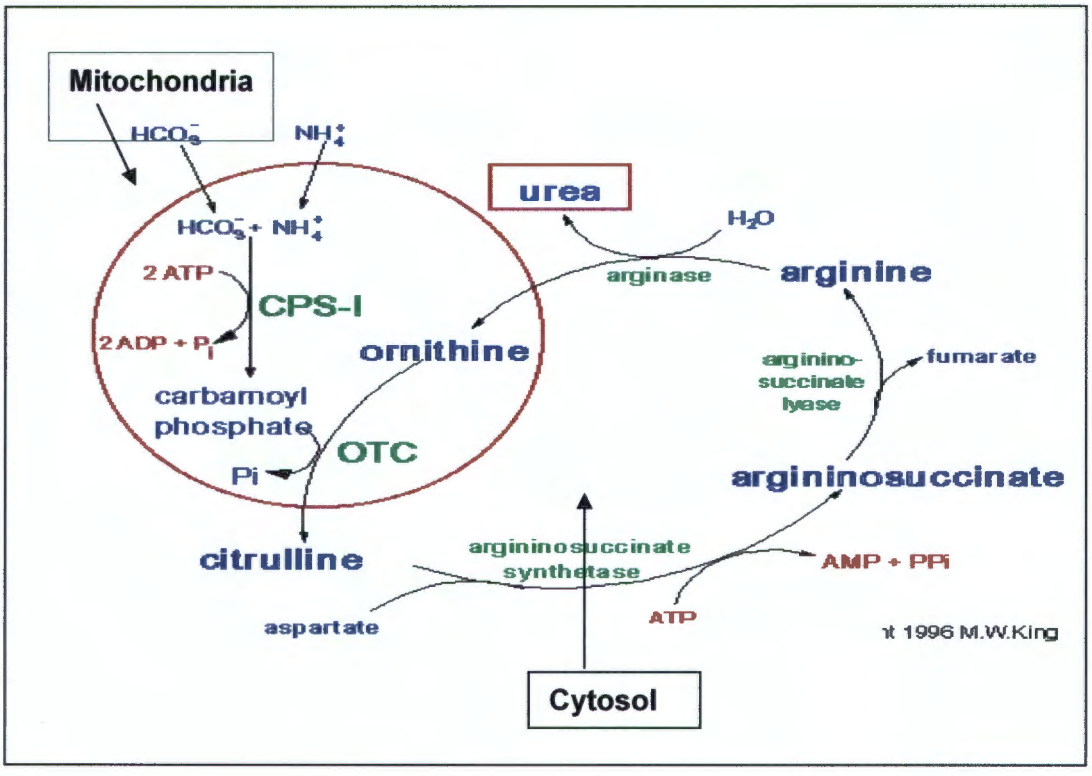


Figure. 1. Schematic illustration of the urea cycle. The reactions of the urea cycle that occur in the mitochondrion are contained in the oval. (Taken from URL 2)

1.2.3 Disorders

Urea cycle disorders are caused by the deficiency or catalytic inactivity of one of the enzymes in the urea cycle. In these disorders, nitrogen accumulates in the form of ammonia, which is highly toxic to the brain, and interferes with energy production and with the normal metabolism of neurotransmitters. Moderate elevations of blood ammonia of 100 to 200 μmol/L (normal < 50 μmol/L) are usually associated with clinical symptoms of lethargy and vomiting, whereas higher concentrations can result in diffuse brain edema, coma, and death. There are two forms in which ammonia is found in body fluids, ionized (NH_4^+), and non-ionized (NH_3). The second (non-ionized) form is more diffusible and therefore more toxic. When taking into consideration the ammonia dissociation constant (pK of 8.95), when the body fluids become more alkaline, the equilibrium will shift towards NH_3, Therefore increasing the toxicity even more. (Tuchman *et al,* 1992)

The exact incidences of urea cycle disorders are under estimated, because many infants die without a definitive diagnosis. (URL. 3)

1.2.3.1 Symptoms and signs

Symptoms for urea cycle disorders appear not only in the neonatal period, but also in early childhood and adulthood. In the neonatal period these include feeding difficulties, lethargy, respiratory distress, impairment of consciousness, vomiting convulsions, and coma. Boys are more commonly affected as a result of OTC deficiency, which is X-linked.

Presentation in the neonatal period is fatal without treatment by dialysis, protein restriction and benzoate administration. Childhood or delayed onset can be seen in both boys and girls, but is less frequent in girls who are carriers for OTC deficiency. Childhood onset symptoms include hyperactive behavior, refusal to eat meat or other high-protein foods, vomiting, lethargy, delirium. In the absence of diagnosis and treatment, coma and death may ensue.

Presentation in adulthood, usually involves stroke-like symptoms, episodes of lethargy and delirium, and are likely to be referred to a neurologist or a psychiatrist. When these symptoms occur in adults, without proper treatment, individuals are at the risk for permanent brain damage, coma and death. Adult onset is more common in women who are carriers for OTC deficiency. (Gordon, 2003 and URL 2)

1.2.3.2 Clinical Presentation and Management

OTC deficiency has been reported at a frequency of about 1/30 000 live births (URL. 4). The phenotypes in males and females have a spectrum of severities. Males with little or no OTC activity present with severe, intractable hyperammonemia beginning several days after birth and proceed rapidly to coma and death. Affected fetuses are protected in the womb by the dialyzing effect of the maternal circulation while the delay in development of symptoms after birth is related to minimal protein ingestion in the perinatal period (Brusilow and Horwich, 1995). Hyperammonemia eventually ensues, given the body's failure to incorporate nitrogen into urea in the presence of protein in the diet.

"Partial OTC deficiency in males (due to mutations, which do not entirely inactivate the OTC gene product), may produce chronic mild hyperammonemia, associated with variable degrees of developmental delay, or may manifest during periods of illness or prolonged fasting, which lead to a catabolic state" (Maestri *et al.*, 1998). Most female carriers are not severely affected, and the severe neonatal course is not seen in female infants. Females do not usually have any symptoms and only experience nausea following high protein meals. Occasional carriers develop lethal hyperammonemia in response to severe metabolic stress, such as surgery. In presenting females, skewing of X-inactivation is the underlying cause. Here, changes in the proportion of active normal X chromosomes versus active chromosome bearing the OTC mutation is the major determinant of residual activity. Lyonisation patterns will differ in female siblings and hence severity in one daughter does not predict severity in another.

Plasma ammonia concentrations between 1000 and 2000μmol/L (normal <50μmol/L) usually accompanies presentation in the neonatal period. Blood urea is usually low and blood gas analysis shows signs of respiratory alkalosis, caused by hyperventilation, stimulated by ammonia effects on the brain. Liver function is generally unaffected with enzyme concentrations in the normal to mildly elevated range (Tuchman *et al*, 1992).

Hemizygous males or heterozygous females having partial enzyme deficiencies normally present later in life with a clinical picture resembling Reye's syndrome. The clinical picture of these patients includes the following; protein intolerance, severe vomiting, lethargy during viral illness, Reye's disease-like episodes and developmental delay. These patients often restrict themselves from food that is high in protein. "Disorders that are treated with valproic acid may unveil a previously undiagnosed urea cycle defect, where the mitochondrial toxicity precipitates hyperammonemia in the patient." (Tuchman *et al*, 1992)

Hair and skin changes (rashes) have been reported and are thought to be a result of arginine deficiency or an imbalance of other amino acids. "Acute hyperammonemia in children and adults even in the range of 200 to 500μmol/L is extremely dangerous, as it can progress rapidly to irreversible brain edema and herniation, whereas newborns with extreme hyperammonemia tend to be more resistant to irreversible brain edema, possibly because of open skull sutures accommodating brain swelling." (Tuchman *et al*, 1992 and Gordon, 2003)

Management

The treatment of urea cycle disorders requires attention to the amount of protein ingested as it is necessary to provide essential amino acids for cell growth and development, and at the same time ensuring that excessive amounts of ammonia are not formed. Protein restriction is thus used in conjunction with scavengers such as sodium benzoate and sodium phenylacetate, which provide alternative pathways for the removal of ammonia. These agents are usually given by way of tube feedings of nasogastric tubes. Most children thus suffer a great deal of distress in taking this medication, which is often accompanied by amino acid therapy. Frequent monitoring of blood ammonia levels is required and frequent hospitalizations are often necessary for control of the disorder. (URL. 3, Gordon, 2003)

Because of the generally poor prognosis for affected individuals, there has been considerable effort placed on developing new treatment approaches. The current focus is on liver transplantation and gene therapy where both have been tried, first in an animal model (the sparse fur *spf*Y mouse) and then in humans (Batshaw et al 1999; Raper et al 2003).

1.2.3.3 Biochemical testing for carrier detection

Two indicators of possible carrier status are an increased plasma glutamine concentration and an increased orotic acid excretion in the urine. There are three biochemical tests that may be helpful in determining carrier status when pedigree analysis is not informative. These tests are; 1) the allopurinol test; 2) the protein tolerance test and 3) the alanine tolerance test. Both the later tests have been abandoned because of the high protein meals are often risks for hyperammonemia and low sensitivities. (Brusilow and Horwich, 1995)

The allopurinol test, which measures orotic acid excretion following allopurinol administration, appears to be the safest and most sensitive in post pubertal girls. The implication of this test for carrier detection in pre-pubertal girls has yet to be determined.

The **allopurinol test** relies on the altered function of hepatocytes in which the mutant allele is expressed. Carbamyl phosphate accumulates and is shunted out of these mitochondria of the cells into the cytosol, where pyrimidine synthesis is stimulated (Fig. 2). One of the metabolites in the pyrimidine pathway, orotidine, accumulates due to the inhibition of orotidine monophosphate decarboxylase, by oxypurinol monophosphate, an allopurinol derivative. The enzymatic block further leads to orotic acid overproduction, which can be detected by urine analysis. In a study by Maestri *et al*, 1998, the sensitivity of this test in 37 women identified, as obligate heterozygotes by pedigree analysis was 0.92. The specificity of this test was 1.00, as none of the normal control women had a positive test. "The sensitivity and specificity of the allopurinol test is substantially lower when orotate is measured; the preferred metabolite to be measured is orotidine." (Maestri *et al*, 1998)

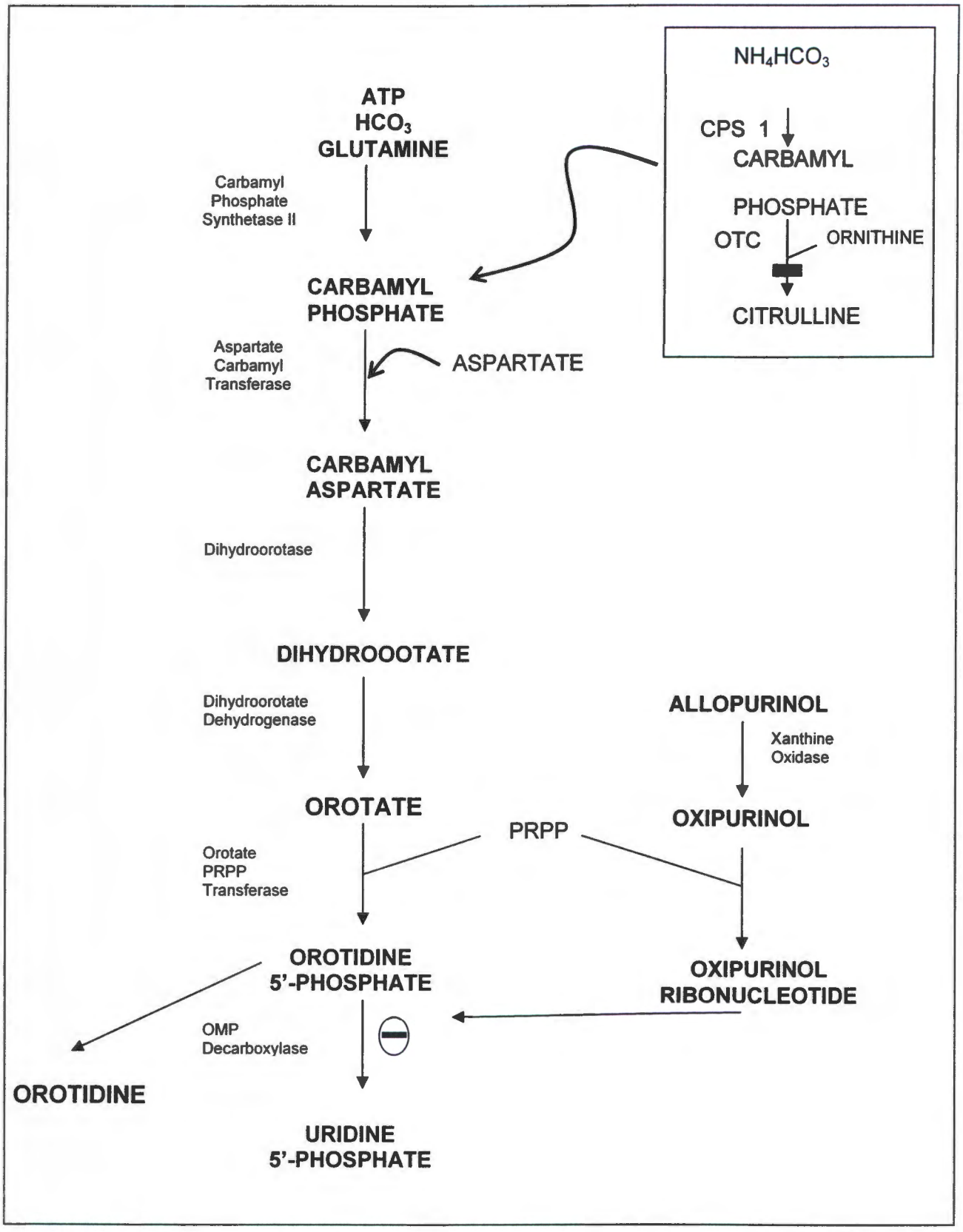


Figure 2. The relation of intramitochondrial and cystolic carbamyl phosphate to pyrimidine biosynthesis and allopurinol metabolism in ornithine carbamyltransferase-deficient hepatocytes. (Brusilow and Horwich, 1995)

The most reliable way to determine carrier status is at the molecular level, by defining the mutation of the proband and testing for a specific mutation in the mother and other female family members. However, one has to be confident that the mutation is a deleterious one and not a polymorphism. In families with an affected female, testing the DNA of the father could help to establish the mutation as a polymorphism, if inheritance from an asymptomatic father can be demonstrated. Finding that the mutation occurred spontaneously in a family member increases the likelihood that it is deleterious. (Tuchman *et al*, 1992, Leibundgut *et al*, 1996)

1.2.3.4 Linkage analysis in Carrier testing and Prenatal Diagnosis

Microsatellite markers have proved to be highly informative markers for genetic mapping and linkage analysis. They are tandem repeats of 2 – 6 bp that occur abundantly and at random throughout most eukaryotic genomes. The length of the repeat regions is usually less than 100 bp and as they are flanked by unique DNA sequence, they can be amplified in vitro using PCR. They display considerable polymorphism due to variation in the number of repeat units and are sufficiently stable to be used in genetic analyses. (Hearne, C.M., *et. al.*, 1992). Linkage analysis can be a highly accurate alternative to mutation-specific diagnosis for families in which a firm diagnosis of OTC has been established, with no mutation found. This method is an indirect method of diagnosis that exploits the tendency of genes and markers that lie close together on a chromosome to be transmitted from parent to child with no recombination. Using linkage analysis for the diagnosis of OTC is relevant, as there are many polymorphisms in and near the OTC gene and recombination in this area can prevent mutations from being transmitted to future generations. In kindreds with more than one affected family member, this method can be used to determine which alleles co-segregated with the disease. When found that the fetus bears the disease-associated allele, it is extremely likely that the fetus is affected. The accuracy of this method is approximately 99%. The main limitation on linkage-based diagnosis is it's use in kindreds with a single affected individual. One third of singleton affected offspring should be new mutants provided that the male and female mutation rates are equal (Bale, A.E, 1999). Increasing use of mutation-specific diagnosis is providing direct data addressing the frequency of new

mutations.

Linkage analysis can also be used to identify an unaffected fetus in kindreds with a single affected person. If the fetus can be shown to have inherited a different OTC locus to the affected child then it is not at risk for OTC deficiency. However, in situations of identity, this linkage analysis becomes uninformative (Bale, A. E, 1999)

1.2.3.5 Laboratory diagnosis of OTC deficiency

Diagnosis of OTC deficiency requires the following has to be considered. Firstly, the blood ammonia level has to be determined. Secondly, plasma amino acid concentrations should be measured, especially citrulline. Thirdly, urinary orotic acid concentrations should be checked and fourthly, a liver biopsy could be done to measure OTC activity. OTC and CPS deficiency can be distinguished from the ASL, ASS and arginase deficiencies by plasma amino acid analysis. Citrulline concentrations in CPS and OTC deficiency are low to zero as the defect in both precedes citrulline synthesis. In contrast, citrulline concentrations are elevated in ASS deficiency while high concentrations of argininosuccinate and its anhydrides are found in ASL deficiency. OTC can be separated from CPS deficiency by the determination of urinary orotic acid or orotidine levels. Both these metabolites are elevated in the former and are absent in the latter (Burton, B.K, 1998).

A flowchart for the differentiation of conditions producing significant hyperammonemia in the newborn is presented in Fig. 3. The timing of the onset of the symptoms may provide important information. Urine organic acid analysis should always be obtained, regardless of whether acidosis is present. Metabolic acidosis is not a typical feature of the urea cycle defects. Plasma amino acid analysis is helpful in the differentiation of the specific defects in this group.

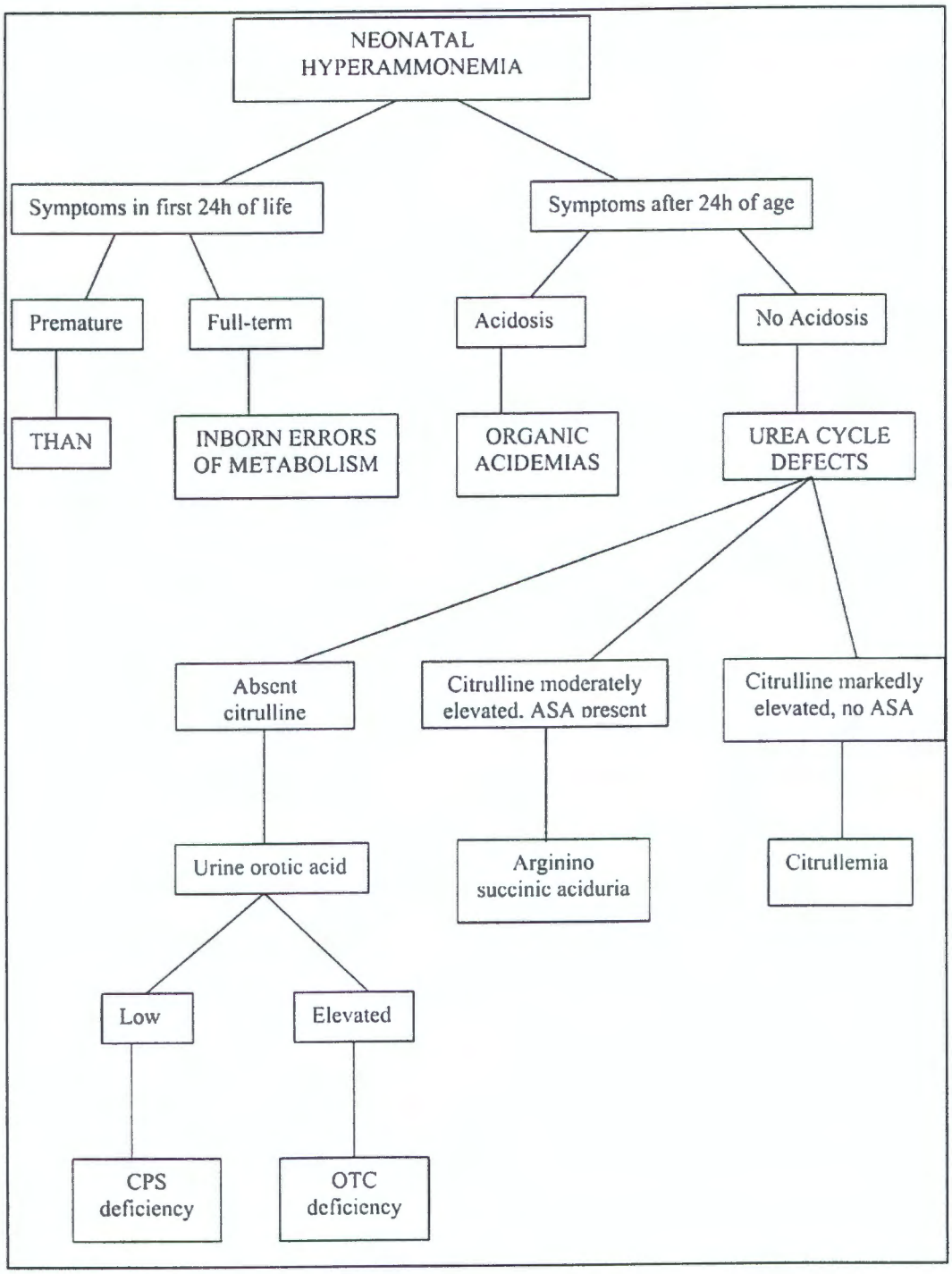


Fig. 3. Flowchart for the differentiation of conditions associated with neonatal hyperammonemia. ASA indicates argininosuccinic acid; CPS, carbamyl phosphate synthetase; THAN, transient hyperammonemia of the newborn. (Burton, B. K., 1998)

1.2.3.6 Molecular genetics

Five urea cycle defects have been described. Four of them, deficiencies of carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinic acid synthetase (ASS), and argininosuccinase acid lyase (ASL), are characterized by signs and symptoms induced by the accumulation of ammonia and glutamine. The fifth defect, arginase deficiency (AG) differs by presenting with progressive spastic quadriplegia and mental retardation, and hyperammonemia that is not as severe or as common in the other four diseases. (Brusilow and Horwich, 1995)

Four of these disorders are transmitted genetically as autosomal recessive traits. One of these defects, Ornithine transcarbamylase deficiency is inherited as an X-linked condition, where mostly boys are affected. (URL. 3)

1.3 OTC deficiency

1.3.1 The OTC gene

1.3.1.1 Mapping

The OTC gene has been mapped to the short arm of the X chromosome on band Xp 21.1 proximal to the Duchenne-Becker muscular dystrophy gene (Fig. 4). Other genes in this proximity are those for retinitis pigmentosa, chronic granulomatous disease, adrenal hypoplasia, glycerol kinase deficiency and Norrie disease. Large deletions in the area around the OTC gene could thus result in contiguous gene syndromes. (Tuchman *et al.* 1992)

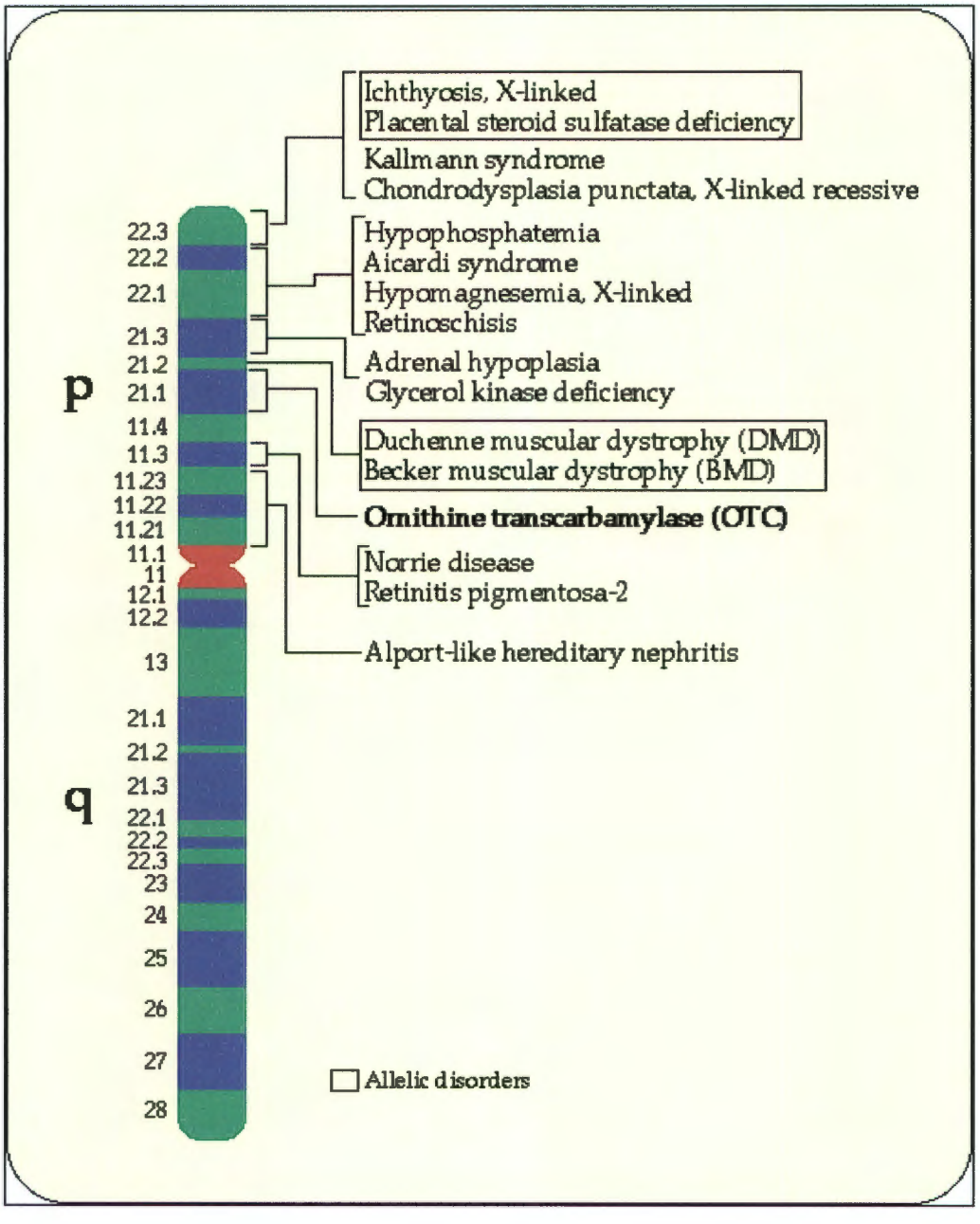


Figure 4. Schematic map of the X chromosome and the genes (p arm) mapped to it. (URL. 5)

1.3.1.2 Organisation and structure

The OTC gene (Fig. 5) was cloned and sequenced in 1984 (Horwich A.L, *et al,* 1984). It spans about 80 kb and contains 10 exons and 9 introns. Exons vary in size from 154bp (exon 5) to 54bp (exon 7) while introns vary from 21.7kb (intron 4) to 80bp (intron 7) in size. (Tuchman *et al*, 1998)

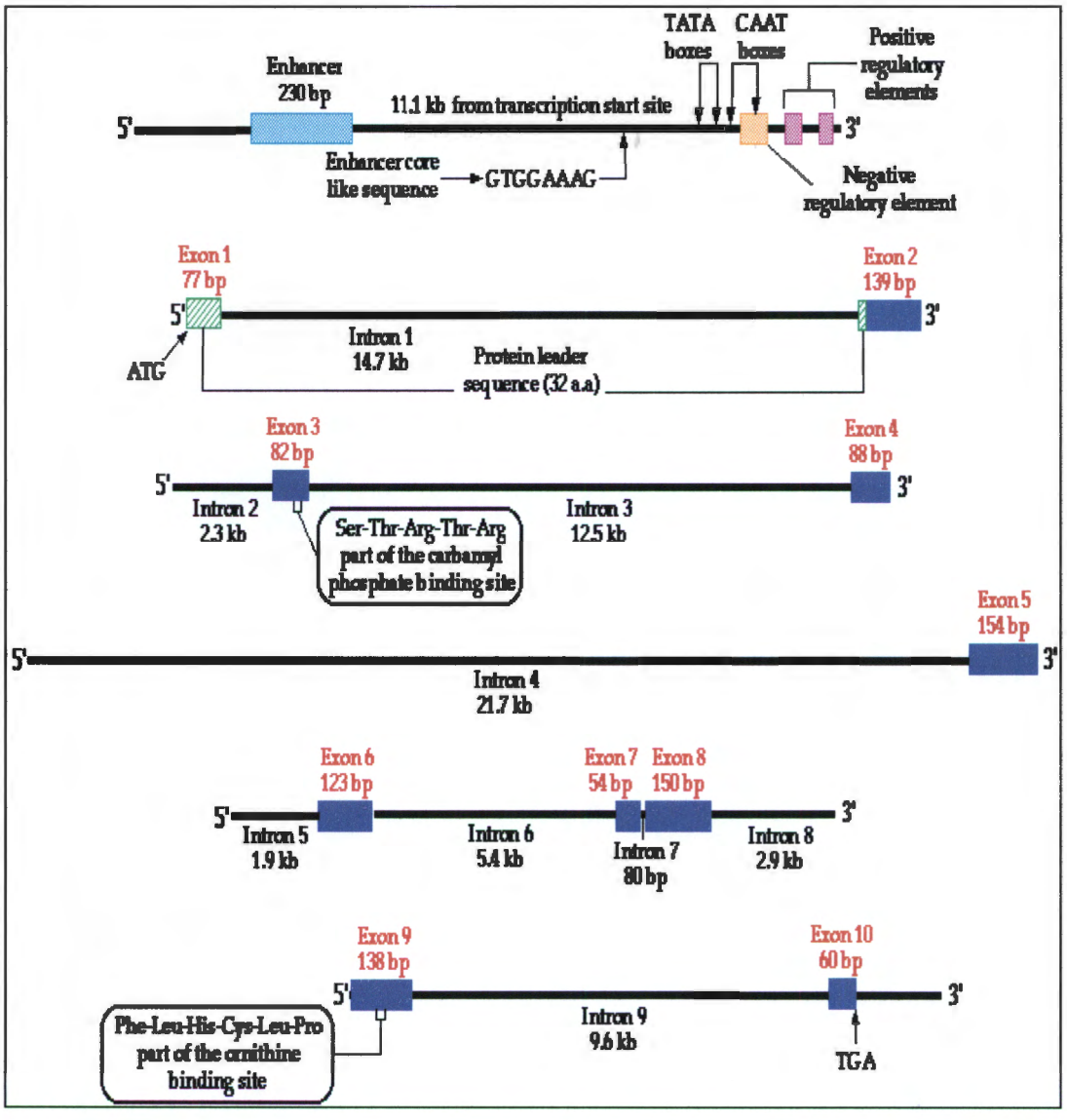


Figure 5. Schematic illustration showing the structure of the OTC gene. (Taken from URL. 6)

1.3.1.3. The synthesis, structure and function of the OTC protein

The OTC enzyme is a trimer with three active sites per holoenzyme molecule. The messenger RNA of the OTC gene contains 1062 translatable bases, which encode the synthesis of a precursor OTC protein that consists of 354 amino acids and a molecular weight of 40 000 daltons. The amino terminus of the OTC protein contains a 32 amino acid leader sequence, which contains eight basic amino acids and no acidic amino acids. The leader sequence is important for binding to the mitochondrial membrane, incorporation into the mitochondrial matrix and intramitochondrial sorting. It is finally cleaved by a neutral protease to produce a mature polypeptide of 36 000 daltons, which is the catalytic unit of the OTC enzyme (Fig. 6) (Tuchman *et al*, 1992). The 150 and 360 untranslated 5' and 3' regions are not translated into proteins.

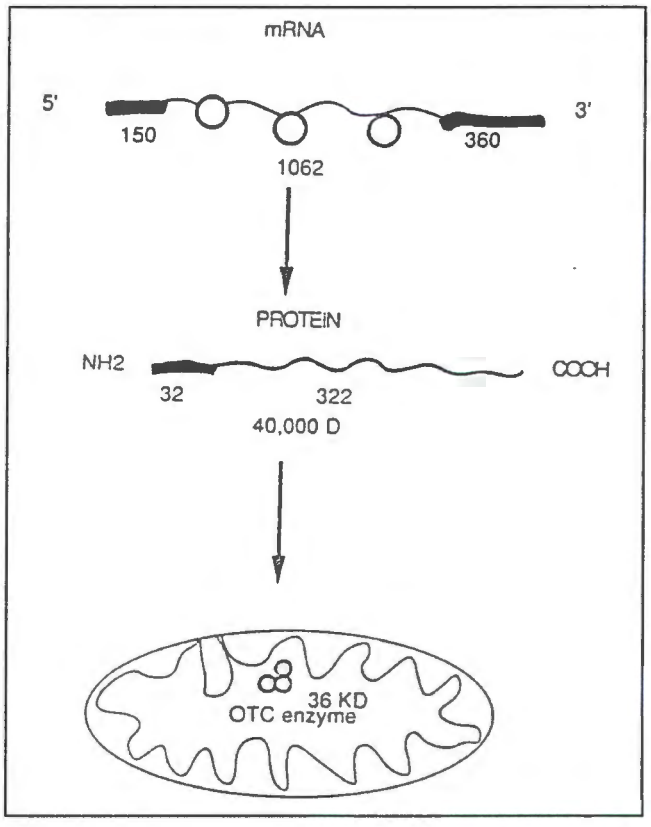


Figure 6. Schematic illustration showing synthesis of the OTC protein. (Tuchman *et al*, 1992)

The OTC molecule comprises of two alpha-domains : the carbamyl phosphate (CP) binding domain, and the ornithine (Orn) binding domain. The molecule is active as

a homo-trimer with the active sites formed at the interfaces between the three monomers. This is clearly shown in the ribbon diagram of the OTC model as derived from recent X-ray crystallographic studies (Shi et al 2001).

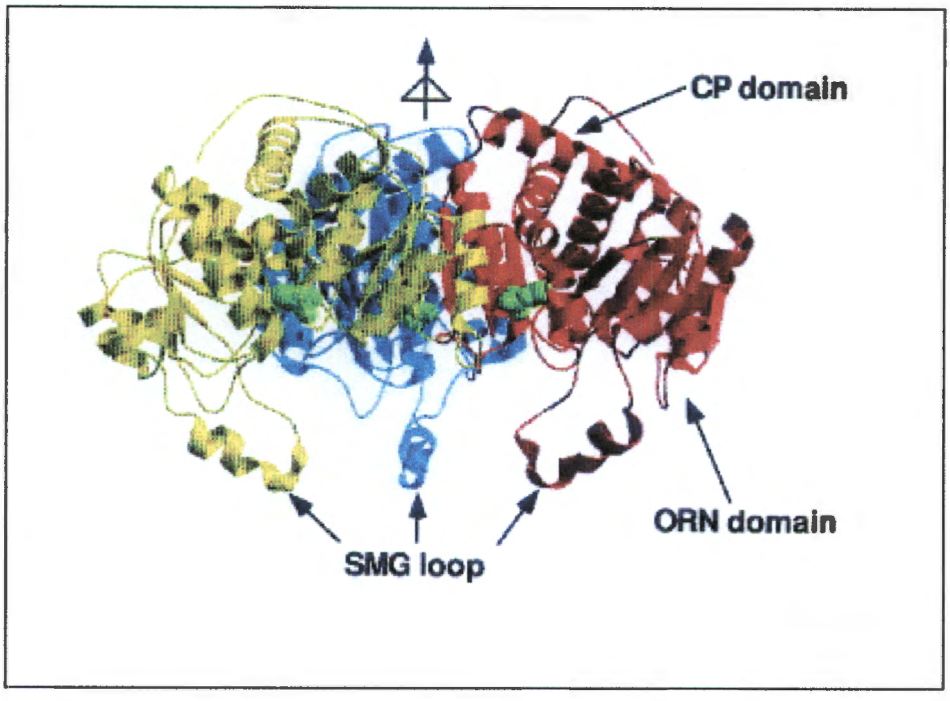


Fig 7. The ribbon diagrams of the OTC homotrimer complex, with each of the monomers represented by a different colour. The CP binding pocket is shown at the base of the cleft while the Ornithine binding pocket appears on the edge. The abbreviation SMG is not clarified in the text.

The OTC gene is expressed mainly in the liver and intestinal mucosa, whereas other tissues show trace or no enzymatic activity. The maximal specific activity of OTC in adult human liver is approximately 100μmol citrulline/gm-wet weight per minute in liver, whereas the specific enzymatic activity in intestinal tissue is about 20% of that found in liver. Problems may be encountered in the enzymatic diagnosis of this disorder in newborns and in patients receiving low-protein diets as OTC activity is low (Tuchman *et al*, 1992).

1.3.2 Mutation database

The OTC mutation database (last updated 2001) (URL 7) lists 230 known mutations in the OTC gene which result in complete or partial OTC deficiency. The majority of mutations found in OTC deficiency, are point mutations. The bulk of these comprise missense mutations, followed by nonsense and splice site mutations. Gross rearrangements, mainly resulting in partial deletions of the gene, account for 10 percent of these cases. The least common mutations are large deletions of the whole gene and contiguous regions of the X-chromosome. Mutations can be found throughout the gene, and almost every family has a unique alteration. The majority of males with neonatal onset of OTC deficiency have mutations in the coding region of the gene or in adjacent splice sites. Direct mutation analysis is less likely to detect mutations in males or females with biochemical studies suggestive of partial OTC deficiency because mutations in this category often lie outside the coding region of the gene (e.g. cryptic splice sites in introns or mutations in regulatory regions) or in a gene other than OTC. (Bale, A. E., 1999)

Approximately 10% of all point mutations involve the CpG dinucleotide of codon 141 with a CGA-->CAA transition producing a deleterious Arg-->Gln substitution in position 109 of the mature enzyme and causing the elimination of a *TaqI* recognition site. The majorities of the remaining mutations in the OTC gene are unique to the affected families and are usually not found in unrelated patients. To date, two mutations have been described in the sequence of the "leader" peptide, 140 mutations have been found in the coding sequence of the "mature" enzyme, and 23 mutations have been discovered in splicing recognition sites. Approximately 20 single base polymorphisms have been postulated to exist by comparing two reported OTC gene sequences; six of these substitutions cause amino acid changes three of which have been confirmed in patients (McCullough, B.A, *et al.* 2000). These collective data can serve for genetic counseling and monitoring in prenatal care.

1.4 Development and Overview of Thesis

OTC deficiency is the most common of the urea cycle defects and is encountered fairly frequently in the Western Cape. The wide experience of the core laboratories at Red Cross Children's Hospital in molecular biology, allowed for several DNA based methods to be applied to mutation analysis in the OTC gene. These included PCR, SSCP analysis, Southern blotting, hybridisation, RFLP and microsatellite analysis. Protocols were optimised for each of these methods and now form part of the diagnostic workup for OTC deficiency in Pathology at Red Cross Children's and Groote Schuur Hospitals.

In total, this study examined four patients with OTC deficiency, which was from four unrelated families. The initial genetic change in kindred 1 proved to be a known polymorphism in the fourth exon of the OTC gene, comprising a single base alteration. Further analysis revealed a novel mutation in the same exon, converting a leucine (CTG) to a glutamine (CAG).

The only sequence alteration detected in patient 2 proved to be a polymorphism comprising a single base change in the second exon of the OTC gene. This point mutation is listed as a polymorphism in the OTC mutation database and is reported at a frequency of less than 10% in a population of European ancestry. The remaining OTC exons from this patient were sequenced but no other sequence alteration was detected.

The genetic defect in kindred 3 proved to be a novel mutation, comprising a large intragenic deletion, which has removed exons 5 to 10. This mutation has not been listed in the international OTC database and is thus novel.

Patient 4 presented with all the features of late onset OTC deficiency, however it was also found that the patient had Klinefelter's syndrome, which significantly complicated the diagnostic workup. Carrier females for OTC deficiency can present clinically, and this is always due to skewed X-inactivation in the liver, where the majority of hepatocytes have the normal X-chromosome inactivated. Skewing of X-inactivation was thus also a possibility in our patient, as Klinefelter's patients have all but one of their X-chromosomes inactivated. There was however a second possibility in our patient and that was the inheritance of two copies of the mutant X-chromosome. Both these possibilities were explored by PCR and microsatellite analysis. We were unable to look for skewing in the liver but were able to show random X-inactivation in white cells; this finding does not however rule out skewing in his liver as the two can be discordant. It was also clear from the microsatellite analysis that the patient inherited two different copies of the X-chromosome, which would thus require skewing in the liver for the presentation of OTC deficiency. A further complicating factor was the finding of normal coding and splice site sequence for each of the 10 OTC exons.

An addition to this thesis was the analysis of flanking microsatellite markers for the OTC gene; three microsatellites (DXS8090, DXS1068 and DXS8113) were selected for analysis. This was prompted by two requests from the parents of the patient in kindred 3 for prenatal diagnosis in subsequent pregnancies. All the microsatellites gave readable gels after PCR and PAGE. Only one however, proved to be informative in this kindred in spite of high reported heterozygosity indexes. This microsatellite was successfully used to detect an affected male fetus in the first prenatal diagnosis and a carrier female in the second pregnancy. The lack of informativeness of two of the three microsatellites in patient 3 was unexpected as the reported heterozygosity indexes in the literature are all >0.68.

The microsatellite markers used in this study have been found to be useful in the indirect analysis of the OTC gene and will aid in the pursuit of carrier detection and

prenatal diagnosis. It was decided to determine the heterozygosity indexes of these three markers in three different population groups in South Africa as these data would allow for a ranking of the microsatellites in order of increasing likelihood of being informative in future OTC diagnostic interventions.

In summary, this study has established an experimental approach to DNA based diagnosis of OTC deficiency and has filled an important void in the diagnostic capability of South African laboratories. Also, no institution in this country has set up the OTC enzyme assays, using liver biopsies, normally utilised to confirm OTC deficiency. These assays are difficult to establish and maintain, and having an alternative diagnostic modality is thus of great benefit. This study has also detected two novel mutations, confirmed the presence of two polymorphisms and demonstrated as in previous published studies that patients with all the clinical and biochemical hallmarks of OTC deficiency, can have a normal coding and splice site sequence and presumably carry a mutation in the promotor, or intronic sequences that affects the transcription of the enzyme or perhaps even a mutation in some other gene impacting on OTC function.

CHAPTER 2

2. Mutation Analysis: Kindreds 1-3 (Methods, Results and Conclusion)

The following format has been used for the presentation of the results.

2.1 Overview

2.2 Ethical considerations.

2.3 Keys to symbols used.

2.4 Pedigrees of kindreds 1, 2, and 3 together with their clinical, sequencing and gene mutation data.

2.5 Methodology used in the protocols.

2.6 Results section includes photographs of informative gels with explanations, schematic presentations and figures to explain the nature and position of the mutations.

2.7 Discussion and Conclusion.

2.1 Overview

This chapter details the procedures and results of a search for mutations in the OTC gene in three unrelated families with phenotypically affected boys and pedigree structures strongly suggestive of an X-linked urea cycle defect. This study was carried out on banked DNA samples as all families had presented during the past 10 years to either of the Red Cross Children's or Groote Schuur Hospitals. Banked cultures of fibroblasts and transformed lymphocytes were also available as another source of DNA. Identification of mutations in families with OTC deficiency enables one to define not only the molecular heterogeneity but also helps to expand knowledge of genotype phenotype relationships. In order to analyse the entire coding region of each mutant OTC gene, the nucleotide sequences, containing each exon plus adjacent intron sequences, were amplified by PCR. The sequences of primers were derived from intron sequences close to the start and end of each exon. This was done in order to reduce the length of flanking intronic sequence co-amplified with the exon, and thus minimise the possibility of finding intronic polymorphisms. The details of the PCR reactions

and the sequences of the primers have been given in the methods and appendix sections.

SSCP analysis of PCR amplified coding sequences is usually the first line approach in the detection of mutations in genes. This is due to the relative simplicity of the technique, its sensitivity, rapidity, and cost effectiveness, all of which render it a popular screening technique. This technique is capable of identifying approximately 80-90% of mutations based on studies of other genes. It is generally accepted that mutations that go undetected often lie close to the ends of the amplified product.

SSCP exploits the fact that the electrophoretic mobility of single-stranded DNA (ssDNA) during nondenaturing polyacrylamide gel electrophoresis (PAGE) is dependent not only on the size, but also on the secondary structure (conformation) of the ssDNA. This secondary structure is stabilised by intrastrand nucleotide interactions, which are determined by the base sequence of the strand. Hence, any base alteration can potentially affect the strand's conformation and therefore it's mobility through and position in the gel. Depending on the resolving power of the PAGE system used and the size of the DNA fragment, a single base substitution can be detected as a conformation dependant shift in position on the gel of one or both of the complementary DNA strands. The mobility shift is conformation dependent and while it signals a sequence alteration it has little comment on the precise nature of the base substitution.

Exons showing abnormal migration patterns on SSCP gels were sequenced on an ABI sequencer using a cycle sequencing approach and fluorescent labelled nucleotides. The fluorescence detectors used are computer controlled and hence the data acquisition is automated.

2.2 Ethical considerations

Informed consent is a fundamental requisite in initiating a research project. This is usually accomplished by a form given to the participant describing the various aspects of the research, manifesting itself as a tool for stimulating a dialogue between researches and the potential participants. The research must project into the future and anticipate the progress of the research, the possible results it may yield, and the ensuing consequences for participants. Specifically the development of new knowledge obtained from the relation between the participant and public should be fully explored. It is therefore important that the consent process include forms that adequately address these issues (Fuller B.P. et al, 1999).

There are important factors that should be considered before initiating a research project and these factors are listed below.

a) **Ethics approval**: The Research Ethics Board will perform a thorough evaluation of the project to ensure that the project respect minimal ethical standards. The guidelines of the project should be both scientifically and ethically acceptable.

b) **Storage of DNA samples**: Sample identification is an important factor to consider. Human tissue samples must be identifiable, traceable, anonymized and be stored for a defined term. Some researchers store samples for five years, whereas others prefer twenty five years to allow another generation to potentially benefit from the information. When initial consent is taken, the group should perhaps include a policy of possibly using the genetic material in other research. The participant does not necessarily have to consent to the subsequent research.

c) **Benefits**: Consent forms should clearly state any anticipated benefits and the participants should not be misled concerning the benefits for themselves. Research should not be offered to any participants, if there is no diagnostic test or any clinical or scientific validation of the research finding that may have an impact on the health of the participant. Some research may not offer any health

benefits to the participants, for example, a genotyping project, or projects that seek to locate a specific gene.

d) **Risks**: Generally there are very few physical risks encountered when participating in basic research, for example, the procedure used to draw the DNA sample when genetic information is collected, there is a risk that this information may be revealed to a third party. This can happen with or without the consent of the participant and may lead to discrimination. The participant must be advised of this possibility.

e) **Confidentiality**: Genetic information acquired about a person over the course of research, requires strict confidentiality. Findings of some genetic research may provide information, not only about the participant's health, but also their families and future progeny.

f) **Communication of results**: When results are being communicated to the participants, it demonstrates the research group's appreciation. It also offers information about the progress of the work as well as personal results.

g) **Genetic Counselling**: Genetic counselling plays a key role in transmitting research results. There are various risks, both economical and social risks associated with the disclosure of results. When these results have impact on the health of the participant, counselling can be psychologically trying for the participant and the family and may alter the participants' decisions regarding future plans. It is therefore essential that the participant understand the implications of the genetic information given. For example, although it may be obvious for the research team, that when a person is a carrier of a gene linked to a disease, it does not mean that the person will develop the disease. (Deschenes M et al, 2001).

This section of ethical issues surrounding consent and DNA banking reveals that many of the ethical and legal controversies of genetic research remain unresolved.

It is therefore imperative not only to seek possible harmonisation of approaches to DNA sampling and banking for research, but also to respect the rights of the participants. This respect is a crucial element for creating a climate conductive both to protection of research participants and to progress in the field of human genetics. Included in this thesis is a copy of the consent form used for the study.

2.3 Keys to Symbols used in family trees.


Normal female, male


Affected female, male


Deceased, affected female, male


Carrier female


Family member of unknown gender


Proband

2.4.1 Kindred 1

Patient 1 (II.5), the second son of healthy parents and brother of a healthy sister and deceased, affected brother (Fig 8) developed fatal hyperammonemia during the first few days of life. The family history was significant in the context of X-linked inheritance as the mother had lost two sons in the neonatal period, one from previous marriage. Patient 1 died twenty days after birth.

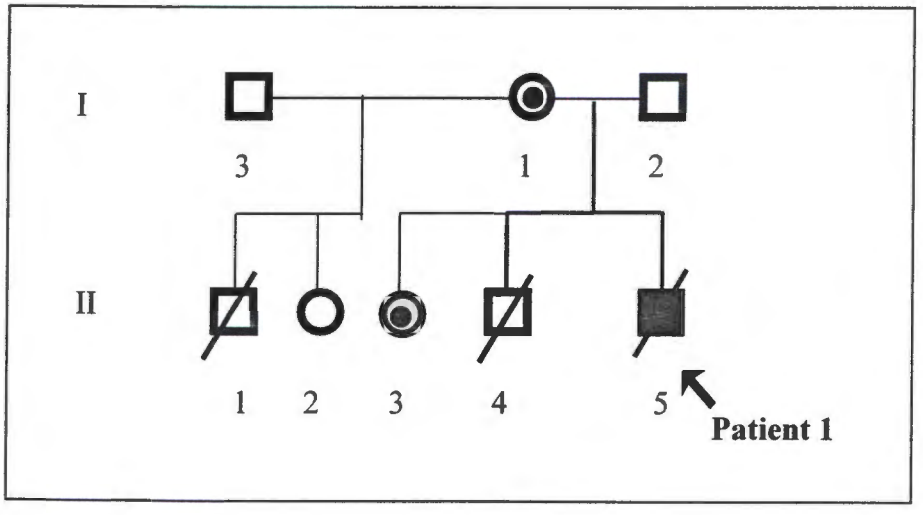


Figure 8. The pedigree of kindred 1 showing the affected male proband, a deceased male sibling and a phenotypically normal sister. Also shown are a deceased half brother and phenotypically normal half sister. For completeness, the genotype findings in this study have also been included in this pedigree.

2.4.2 Kindred 2

Patient 2, (II.5) was the fifth son born of healthy parents, and brother of four deceased, boys (Fig 9). He was originally diagnosed as having CPS deficiency, but the family pedigree strongly indicated an X-linked pattern of inheritance and a likely OTC defect. An amino acid analysis of plasma specimens confirmed the absence of citrulline, more important there was an increase of orotic acid excretion in the urine.

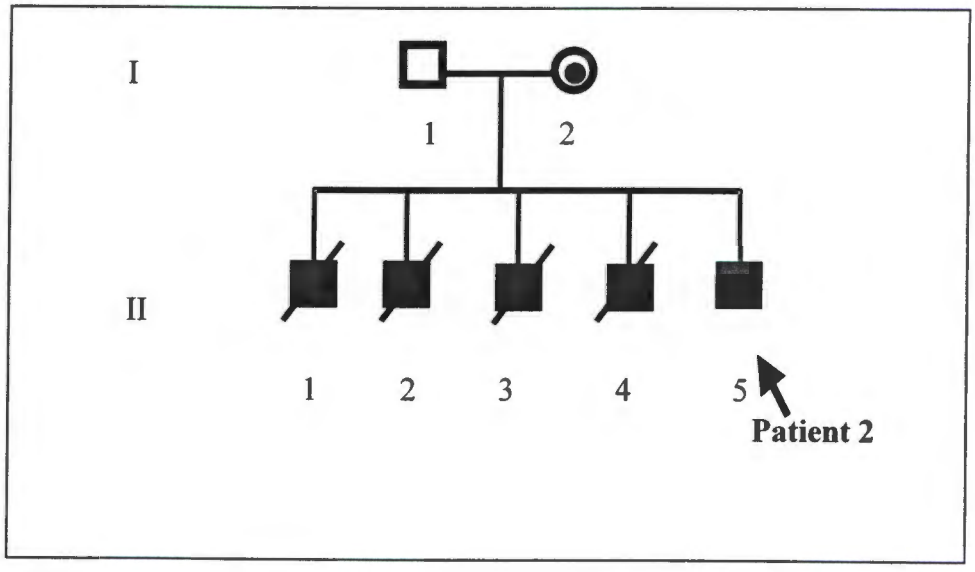


Figure 9. The family tree of kindred 2. Shown are the parents, the proband and 4 deceased male siblings. For completeness, the genotype findings in this study have also been included in this pedigree.

2.4.3 Kindred 3

Patient 3 (II.4), the fourth son of healthy parents and brother of two deceased, affected siblings and one normal brother (Fig 10), developed fatal hyperammonemia a month after birth, and died in early infancy. Urinary orotic acid levels were considerably elevated in this neonate. This finding, coupled with the family history of multiple early male deaths was strongly suggestive of an X-linked pattern of inheritance and specifically for OTC deficiency.

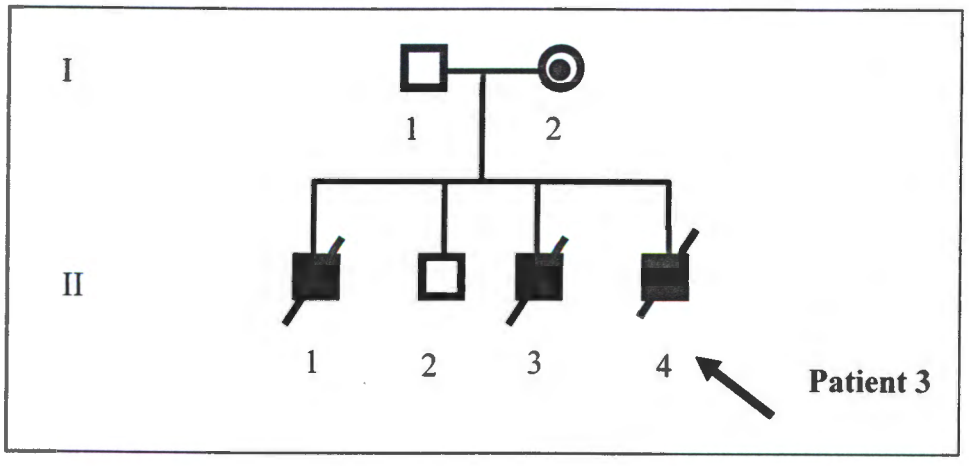


Figure 10. The family tree of patient 3 (II.4) showing two affected male siblings, one normal male sibling and both parents. An arrow indicates the proband. For completeness, the genotype findings in this study have also been included in this pedigree.

Chapter 2.5: Methodology

The major steps followed in the mutation analysis of the OTC gene are given in the following flow diagram. Please refer to appendix for details concerning the reagents.

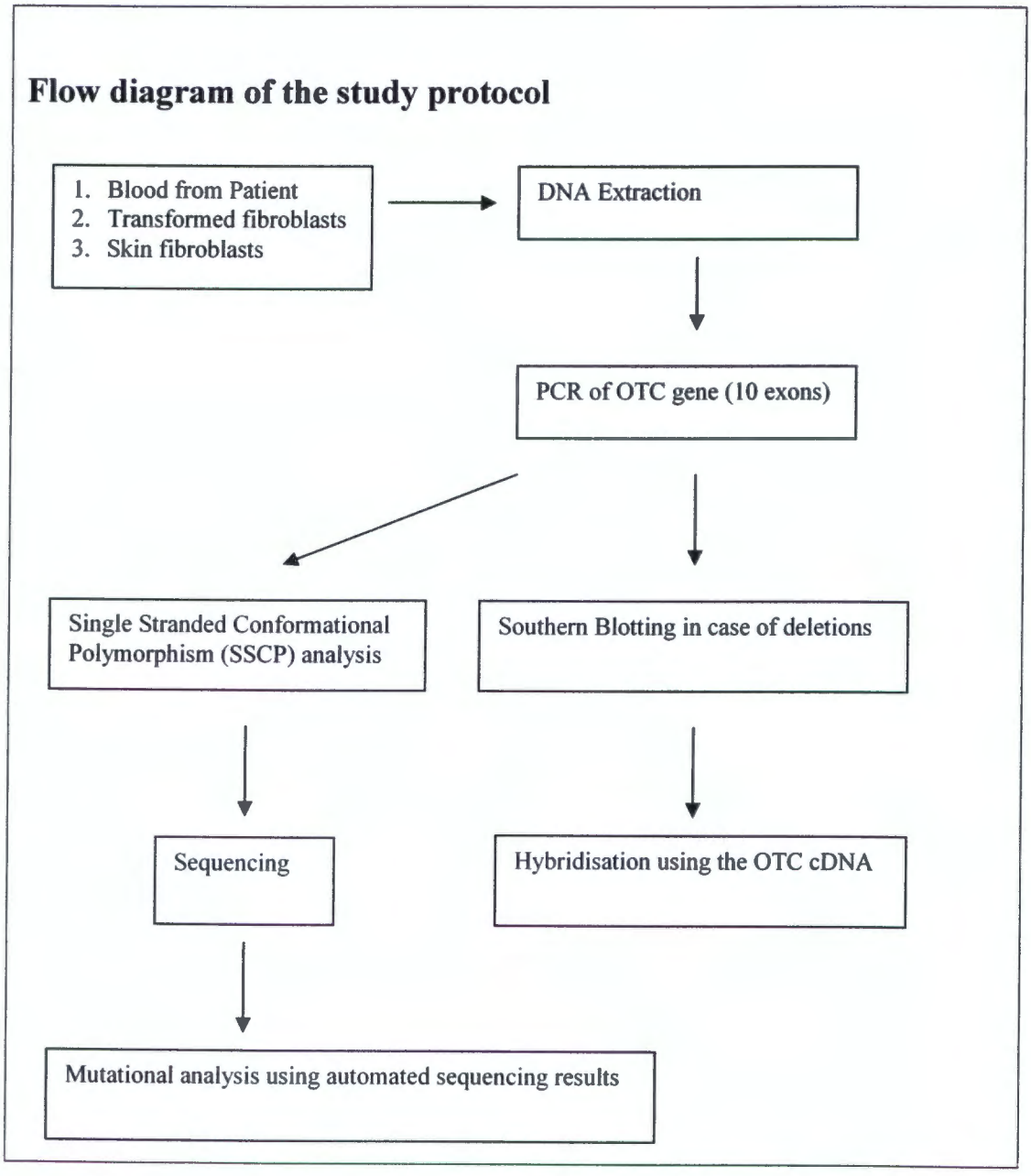


Figure 11. Flow diagram representing the methodology used in the mutational analysis of the OTC gene.

The DNA extraction method of Parzer *et al* 1991 was found to be optimal for heparinized blood and the non-enzymatic salt method of Lahiri *et al* 1991 was optimal for EDTA blood. All the DNA samples were observed to be intact and of high molecular weight on electrophoretic analysis.

2.5.1. Genomic DNA extraction protocols

2.5.1.1. Whole blood extraction method

Whole blood was collected in a Vacutainer tube containing 100 µl of 15% EDTA (1 mg/ml). Blood (5 ml) was transferred into a 15 ml centrifuge tube containing 5 ml of low salt buffer. Nonidet P-40 (125 µl) (NP-40, Sigma) was added and the tubes were mixed well by several inversions to lyse the cells. Samples were then centrifuged for 10 minutes at 2200 RPM at room temp. (Beckman centrifuge). Supernatant was decanted and the nuclear pellet (the small pellet at the very bottom of the tube) was washed in 5 ml of TKM1 buffer. Samples were centrifuged as before. The pellet was gently resuspended in 0.8 ml of high salt buffer and 50 µl of 10% SDS. The whole suspension was mixed thoroughly by pipetting back and forth several times, incubated at 55°C for 10 minutes. To the tubes 0.30 ml of 6 M NaCl was added and mixed well. Samples were centrifuge for 5 min at 12000RPM, in a microcentrifuge. The supernatant containing the DNA was saved and the precipitated protein pellet at the bottom of the tube was discarded. To the supernatant 2 volumes of 100% ethanol was added at room temperature. The tubes were inverted several times to precipitate the DNA in the solution. Precipitated DNA strands were put in a microfuge tube containing 1 ml of ice-cold 70% ethanol and microfuged for 5 min at 12000RPM at 4°C. Pellet was dried in a Speed-vac and the DNA was resuspended in 0.5 ml of TE buffer and incubated at 65°C for 15 min to allow for complete dissolution.

2.5.1.2. Frozen blood extraction method

Frozen blood (5 ml) was thawed at 37°C and mixed with 50 ml of cell lysis buffer (blood: lysis buffer ratio of 1:5) in a Beckman tube and incubated for 15 min. on ice. Samples were centrifuge for 10 min. at 6800g and 4°C and the supernatant was immediately decanted. The pellet was resuspended in 50 ml of wash buffer

and centrifuge for 10 min. at 6300g and 4°C. Again the supernatant was immediately decanted. The remaining pellet (which should be almost white) was mixed with the following reagents in this order: 350 µl of 20% sodium sarkosyl, 250 µl of 7.5 M-ammonium acetate, 3.5 ml of 6 M-guanidinium chloride and 125 µl of proteinase K (10mg/ml). Beckman tubes were incubated for 15 minutes at 60°C or until the solution was clear and immediately cooled to 0°C. DNA was precipitated by the addition of 10 ml ice-cold ethanol. Gelatinous DNA was spooled on a hooked glass rod and released into 500 µl of TE buffer and allowed to dissolve completely in the tube by incubating it for 1 hour at 37°C or overnight at room temperature.

Generally under these conditions the DNA is not completely soluble. However the insoluble material can easily be separated and removed by centrifugation (10-15 s) at 1000g in a Microfuge centrifuge.

The concentration and purity of the DNA was determined by measuring the OD_{260}/OD_{280} ratio and by viewing the extracted DNA on agarose gel electrophoresis.

2.5.1.3. Bloodspot extraction method

Whole blood (250µl; EDTA or heparinized) was pipetted onto sterile filter paper, allowed to dry overnight, sealed in a plastic sleeve and stored at -20°C for future studies.

A 3mm x 4mm square of the bloodspot was cut out with a sterile blade and dropped into a 1.5 ml Eppendorf. Methanol was added to the tube to cover the square and was left to stand for 5 minutes and then allowed to dry completely. Boiling each piece in 50 µl sterile water for 15 minutes liberated the DNA. The mixture was then centrifuged for 10 minutes at 10 000rpm's and kept on ice. On average 10-20 µl of the extract was used for PCR reactions with a final volume of 50 µl.

2.5.2. Determination of DNA concentration

2.5.2.1. By Spectrophotometric readings A_{260}

A 1: 100 dilution of DNA was prepared in distilled water (10 µl in 990 µl) and the absorbance readings taken at OD_{260} and OD_{280} on a spectrophotometer. The A_{260}/A_{280} ratio gives an indication of the purity of the DNA preparation. A pure sample would have a ratio of 1.8 - 2.0.

The DNA concentrations were calculated as follows:

For double stranded DNA 1 OD = 50 µg/ml

For single stranded DNA 1 OD = 33 µg/ml

DNA concentration = OD_{260} x 50 µg/ml (or 33 µg/ml) x dilution factor

2.5.2.2. By agarose gel electrophoresis

A 1 µl DNA sample was mixed with 3 µl of 6 x loading buffer and the sample made up to 10 µl with distilled water. This was run on a 0.8% agarose gel in 1 x TAE buffer at a constant voltage of 80Volts/cm for 30 minutes. A high molecular weight marker was run on the gel for comparison to the genomic DNA.

Ethidium bromide is a florescent dye that intercalates between the DNA strands, allowing visualization of the product under a UV transilluminator. Smearing would indicate degradation of the DNA. Quantitation of the DNA can be achieved by comparing the intensities of the bands of the different genomic DNA or PCR samples to the intensity of DNA bands of concentration.

2.5.3. Polymerase Chain Reaction (PCR)

2.5.3.1. Primer design

All primers were designed using the OLIGO™ computer program. Here sequence length, GC content, melting temperatures and oligo self-complimentarity were easily compared.

2.5.3.2. PCR amplification

The following quantities were used per reaction mix in a 50 µl sample tube: 4 µl 2.5mM dNTP's, 4.5 µl 10 x Promega buffer, 2.0 µl 25mM $MgCl_2$, 1.0 µl 20pM forward primer, 1.0 µl 20pM reverse primer, 31.5 µl distilled water and 1.0 µl 0.5 µg/µl DNA. Control samples with no DNA were added as well. PCR mixtures were overlaid with mineral oil (45 µl) to prevent evaporation and condensation of the samples. Tubes were placed in a thermocycler and subjected to a "hot start" by the addition of the 5 µl enzyme mix after the first 95°C for 3 minutes step in the PCR cycle. An enzyme mix was prepared to contain the following quantities per reaction: 0.5 µl Taq DNA polymerase (5U/µl), 0.5 µl 10 x Promega buffer, 4.0 µl distilled water.

2.5.3.3. Thermocycling

An appropriate annealing temperature profiles was chosen for each set of primers and the individual annealing temperatures were programmed into the thermocycler (Appendix).

The following template programme was used:

Step i. Denaturation at 95°C for 3 minutes

This was set for 1 cycle

ii. denaturation at 95°C for 30 seconds

iii. annealing at __°C for 1 minute

iv. extension at 72°C for 1 minute

This was repeated for 10 cycles

v. denaturation at 92°C for 30 seconds

vi. annealing at __°C for 1 minute

vii. extension at 72°C for 1 minute

This was repeated for 23 cycles

vii. Final extension at 72°C for 5 minutes

2.5.3.4. Agarose gel electrophoresis

An aliquot (5 µl) PCR product was mixed with 2 µl of 6 x loading dye and electrophoresed on a 2.0% agarose gel in 1 x TAE buffer at a constant voltage of 100Volts/cm for 45 minutes. A high molecular weight marker was prepared and run on the gel for size determination of the PCR product.

2.5.4. Single stranded conformation polymorphism (SSCP)

2.5.4.1. Preparation of plates

The surfaces of both plates were thoroughly washed with detergent and 70% ethanol and allowed to dry completely. Siliconizing agent was applied (in a fume cupboard) to one plate and vigorously rubbed onto the surface. The plate was allowed to stand in the fume cupboard for a few minutes. A plate glue mix, containing 90 µl 10% acetic acid and 3 ml of the plate glue stock, was thoroughly rubbed onto the surface of the other plate which was allowed to stand for 3 minutes. Excess plate glue was rubbed off with 100% ethanol. The ethanol wipe was done twice to ensure the removal of the plate glue, to prevent the plates sticking together after electrophoresis, due to the diffusion of the glue through the gel. The plates were assembled and secured with the aid of bulldog clips and with spacers (0.4 mm) separating the two plates.

2.5.4.2. Preparation of polyacrylamide (PAGE) gels

2.5.4.2.1. Gel type 1		2.5.4.2.2. Gel type 2	
The following volumes were added to make gel type1:		The following volumes were added to make gel type 2:	
40% Acrylamide stock (39.6:0.4)	18.75ml	10% Acrylamide (30:0.8)	27 ml
0.6 x TBE	9ml	5 x TBE	8 ml
Pure Urea	5.6 g	glycerol	4 ml
10% APS (Ammonium Persulfate)	400 µl	10%Urea	12 g
Distilled water	40 ml	distilled water	35 ml
TEMED	25 µl	10%APS	800 µl
		TEMED	80 µl
Total	**~70 ml**	**Total**	**~75 ml**

TEMED was added last as this caused polymerisation of the gel mix. The solution was gently mixed, taken up in a 50 ml syringe and poured between the two plates which were lying on a flat surface. A well-forming comb was inserted at the top of the two plates and bulldog clamps were used to hold the two plates firmly together until polymerisation was completed. This process occurred in about 90 minutes. The gels were then ready for use.

2.5.4.3. Sample preparation and electrophoresis

An aliquot (5 µl) of the PCR product was mixed with SSCP loading dye (5 µl). Tubes were incubated at 95°C for 3 minutes to denature the DNA and immediately put on ice. Combs were removed from the gel to form the wells. The gel was placed into the electrophoresis tank and filled with TBE buffer. The wells were cleaned of urea with the aid of a syringe. Two different gel conditions were followed. Gel type 1 was run at room temperature at 6W overnight. Gel type 2 was run in the cold room, which was at 4°C, overnight. Gel type 2 had an

initial run of 75W for 5 minutes and then the wattage was decreased to 9W for overnight.

2.5.5. Silver Staining

2.5.5.1. Method 1

The silver stain relies on the change in pH and the precipitation reaction of the silver ions onto the DNA in the gels. Ammonia was used for gel impregnation and a dilute acid solution of formalin was used for image development, which was brought about by the change in pH from basic to acidic which caused the silver ions to deposit onto the DNA thereby forming insoluble silver salts.

The two plates were disassembled and the plate containing the gel was put in a gel tray for silver staining. This plate was covered with 2 litres of distilled water and gently agitated for 30 minutes following which water was discarded and the ammoniacal silver solution was added. The tray was covered to limit evaporation and slowly agitated for 20 minutes. The silver solution was poured off. Distilled water was added to the tray to cover the gel and left to shake for 30 seconds. Water was discarded and the developing solution (citric acid, formalin) added to the tray. The reaction was stopped when faint bands became visible on the gel. The developing solution was discarded and water was added to the tray. When the bands were clearly visible a photo of the gel was taken and the plates soaked in 0.5M NaOH to remove the gel from the plate following which the plates were given a quick rinse and stored for the next gel.

The high sensitivity of the silver stain technique renders it susceptible to staining artefacts and impurities. The use of deionised water is critical for the success of the procedure and the conductivity of the distilled water should be measured before use.

2.5.5.2. Method 2

Following electrophoresis and disassembly the plate containing the gel was put in a gel tray for silver staining. The gel was then covered with 2 litres of distilled water and gently agitated for 1 minute. The water was then discarded and solution1 was added. The tray was slowly agitated for 10-15 minutes. Solution 1 ($AgNO_3$) was decanted and distilled water was poured into the tray and left to shake for 2 minutes. The water was then discarded and the second solution was then added to the tray. Agitation was continued for 10-15 minutes or until the bands were clearly visible. Solution 2 (NaOH / NaBH4 and formaldehyde) was then discarded and water was added to the tray and left to agitate for 1 minute. Thereafter the water was discarded and solution 3 was added to the tray to sharpen the bands. Solution 3 (Na_2CO_3) was then discarded and the gel was then reimmersed in water. The gel was then viewed under a light box. A photograph of the gel was taken and the plates were soaked in 0.5M NaOH to remove the gel from the plate. The plates were given a quick rinse and then stored for the next gel.

The exons showing a band shift were then amplified through PCR, purified and submitted for sequence analysis.

2.5.6. Sequence analysis

2.5.6.1. Purification of DNA

2.5.6.1.1. Ammonium acetate precipitation

To the PCR product 1/2 volume of cold 7.5M ammonium acetate was added and the tubes mixed. Cold absolute ethanol (2.5 volumes) was added and mixed. The tubes were then placed at -70°C for 30 minutes to precipitate DNA. This was recovered by centrifugation for 20 minutes in microfuge at 14000rpm's. The supernatant was discarded and the pellet washed with 70% cold ethanol and centrifuged for 10 minutes at 14000rpm's. The supernatant was discarded and the pellet allowed to dry for 5 minutes in a speedvac. The DNA pellet was then dissolved in 50 µl TE or distilled water.

2.5.6.1.2. By agarose gel electrophoresis

The 50 µl of DNA was then mixed with 6 µl 40%sucrose and pipetted into the wells of the 1% low melting point agarose gel and electrophoresed in 1 x TAE buffer at a constant voltage of 60V for 45 minutes. An appropriate molecular weight marker was run to be able to track the migration of the bands in the gel. Ethidium bromide was not used to stain the DNA. The separated bands were viewed under the UV transilluminator and the appropriate bands were cut out with a sterile blade, cutting as little of the agarose around the band as possible. The excised bands were then weighed and dropped into a 1.5 ml Eppendorf.

2.5.6.1.3. Qiagen II gel extraction kit

The bands were then purified with the aid of the Qiagen II Gel Extraction Kit. A volume of 0.3 ml QX1 solubilization buffer was added per 100 mg of gel. After solubilization 10 µl of Qiaex beads was added to the Eppendorf and the suspension was then incubated at 50°C for 10 minutes, with vortexing at 2 minute intervals. The samples were then centrifuged in a microfuge for 30 seconds. Supernatant was carefully removed and the pellet in 0.5 ml QX1 buffer to get rid of residual agarose. The pellet was then resuspended in 0.5 ml equilibration buffer PE, washed twice to the remove the salt contaminants and air dried for 15 minutes until it became white. DNA was eluted from the beads by the addition of 20 µl TE buffer, vortexing, incubation at room temperature for 5 minutes, which was then followed by centrifugation for 30 seconds. The supernatant, now containing the DNA, was carefully removed. This elution process was repeated twice. The purity and concentration of the DNA was determined through spectrophotometric readings and 0.8% agarose gel electrophoresis.

2.5.6.2. Automated Sequencing

For the sequencing of PCR DNA template a concentration of 180-250 ng was required per reaction. The template (250 ng) and the primer (20 pM/µl per reaction) were supplied in a single tube, which was made up to a total volume of 12 µl with distilled water for each primer.

Sequencing was done on an ABI 373 stretch automated sequencer using 4-colour fluorescent technology. Cycle sequencing using dye-labelled terminators was used. It is a rapid and convenient method of performing enzymatic extension reactions for DNA sequencing. The enzyme used was Amplitaq DNA polymerase FS that was developed specifically for fluorescent cycle sequencing. It is a mutant form of *Taq* DNA polymerase which has essentially no 5'-3' exonuclease activity and has a drastically reduced discrimination for dideoxynucleotides. Essentially the symmetric double-stranded PCR templates were purified prior to sequencing and then subject to 25 rounds of cycle sequencing reaction with sequencing primers and terminator premix (A-dye, C-Dye, G-dye, T-Dye, dGTP, dATP, dCTP, dTTP Tris-HCl pH 9.0, $MgCl_2$, thermostable pyrophosphatase and Amplitaq FS) followed by ethanol precipitation to remove unincorporated nucleotides. The samples were resuspended in formamide, denatured and loaded onto an 4.25% polyacrylamide (Ameresco) gel and electrophoresed for 16 h at 1000 v, 32 watts. Automated analysis was performed using ABI sequencing analysis software version 2.0.12.0.1.

2.5.6.3. Restriction digestion

2.5.6.3.1. Primer design

Often a mutation can be verified with the aid of restriction enzymes as the sequence alteration in the DNA destroys or creates a cutting site for one of these enzymes.

The mutation detected in patient 2 destroyed a restriction site in the PCR product from the mutant allele.

2.5.6.3.2. PCR product amplification

The PCR amplification protocol was followed as in 2.5.3.1. – 2.5.3.4.

2.5.6.3.3. PCR product digestion

Dde I (10U/µl)-10 x Buffer H incubated at 37°C (refer to Fig 19)

The restriction digest contained the following quantities per 20 µl sample:

PCR product	5 µl
10 x Buffer	2 µl
Enzyme (10 U/µl)	2 µl
distilled water	11 µl
	20 µl

The undigested samples were used as controls and contained no enzyme but instead contained equal volumes of distilled water as replacement. The PCR products were incubated with the enzyme and buffer combination at 37°C. Digestion was allowed to occur overnight and the products were run on a 20% PAGE gel.

2.5.6.3.4. Electrophoresis and visualisation

The following gel mix for a 20% PAGE gel was added to an Erlenmeyer flask:

30% acrylamide (29:1)	33.3ml
5 x TBE	10ml
distilled water	6.3 ml
10% APS	400 µl
TEMED	25 µl
	50 ml

Two small plates (15 cm x 15 cm) were a cleaned and assembled. Two spacers of sizes 0.75 mm were used to separate the plates. A 0.75 mm comb was used to form the wells. After polymerisation the comb was removed and the gel was placed in the tank in 1 x TBE buffer.

10µl of digested product was mixed with 2 µl of 6 x loading dye and loaded into the wells. The gel was electrophoresed at 220V for 3 hours. The products were

visualised with the aid of a silver stain or an ethidium bromide stain (9 µl EtBr mixed with 125 ml 5 x TBE).

2.5.7. RFLP and Southern Blot Analysis

2.5.7.1. Digestion of Genomic DNA

A 10 µg amount of each sample (Patient DNA and Control DNA) of genomic DNA was incubated with 4 µl (*BamHI*, *HindIII*, and *PvuII*) restriction enzyme and 10 µl 10X Incubation buffer in a total volume of 100 µl ddH_2O at 37°C overnight.

2.5.7.2 Precipitation of Digested Product

Cold absolute ethanol (2.5 volume 100%) was added to the Restriction digested product and mixed. The tubes were then placed at -70°C for 30 minutes to precipitate DNA. The tubes were then centrifuged for 20 minutes in microfuge at 14000rpm's. The supernatant was then discarded and the pellet was washed with 70% cold ethanol and centrifuged for 10 minutes at 14000rpm's. The supernatant was discarded and the pellet was allowed to dry for 5 minutes in a speedvac (An instrument used to dry specimens using liquid N_2. The DNA pellet was then resuspended in 15 µl distilled water to dissolve.

2.5.7.3. Gel Electrophoresis of Digested Product

The digested DNA samples were electrophoresed in 1X TAE buffer, at 30V for 16 hours(overnight) on a large 0.8% agarose gel in 150 ml of 1X TAE containing 2ul 10 mg/ml ethidium bromide. A high molecular weight maker was run on the gel for comparison of the digested bands. Ethidium bromide staining allowed the DNA to be visualised using a UV transilluminator. A photograph of the gel alongside a ruler was taken, under UV light, for reference, allowing for calculation of molecular weights of individual bands ultimately observed on the autoradiograph, according to the distance travelled on the gel.

2.5.7.4. Transfer of DNA onto nylon membrane.

Gloves were worn as protection against acidic and alkaline solutions and to prevent membrane contamination. The gel was removed from the tray and placed in a glass dish. Ten gel volumes of 0.25M HCl (approximately 1.5 L) were added to the glass dish, which was incubated on a platform shaker for 30 minutes at room temperature. This step results in partial depurination of the DNA fragments, which lead to strand cleavage. The length reduction improves the transfer on longer molecules.

(Depurination was stopped after 10 minutes when the xylene cyanol and bromophenol blue dyes, have changed to green and yellow respectively). The HCl was poured off and the gel was rinsed with distilled water. Ten gel volumes of 0.4 M NaOH was added to the gel in the glass dish, and was incubated as previously described. This was the denaturation step.

A clean piece of plastic wrap was placed on the bench top. A clean glass plate was placed in the centre of the plastic wrap. Once the gel has been washed in 0.25 M HCl and 0.4 M NaOH, it was carefully transferred onto the glass plate. The edges of the plastic wrap were folded up to cover all four outer edges of the gel. Care was taken to ensure that edges were not exposed and that the plastic did not cover the outer lanes. The Hybond membrane was cut to the size of the gel, and wet in 0.4 M NaOH. It was then laid over the gel and the bubbles gently pressed out. Care was taken to handle the membrane with gloves and forceps to avoid contamination by fingerprints. The top right-hand corner was cut for orientation. Three pieces of similar size filter paper that have been soaked in 0.4 M NaOH were placed on top of the membrane. A 2" stack of folded paper towel was placed on top of the filter papers. A weight (Perspex tray and a bottle) was placed on top of the paper towel. This apparatus was left at room temperature for approximately 16 hours overnight, for the transfer of the DNA onto the membrane to occur by trans gel buffer flow.

2.5.8. Preparation of cDNA Probe

(The cDNA was used as a probe for the hybridization step)

2.5.8.1. Preparation of cDNA clone

Dr. Wayne Fenton, Yale University, supplied the clone pH0731 (5-10 μg).

PH0731 an ethanol precipitate was resuspended in 100 μl sterile TE buffer to yield a concentration of 0.05 μg/μl or 50 ng/μl. This was aliquoted into 5 tubes and stored at –70°C. For PCR, a 1:1000 dilution was made to yield a concentration of 0.05 ng/μl.

2.5.8.2. PCR of OTC cDNA (pH0731)

The following OTC primers were used to amplify the whole cDNA:

OTC Exon1 Forward: 5'-AGT TTT CAA GGG CAT AGA ATC-3'

OTC Exon10 Reverse: 5'-TCA TTC TGT TAC TGA AGA ACA TTG C-3'

The PCR amplification protocol was followed as in 2.5.3. These primers used a final $MgCl_2$ concentration of 1.5 mM and an annealing temperature of 55 / 52 °C. (See section 2.5.3)

2.5.8.3. Concentration of pooled PCR product (cDNA)

Pooled PCR products of 10 samples were concentrated by adding 1/2 volume of cold 7.5 M ammonium acetate were added to the tubes. To this 2.5 volume 100% cold absolute ethanol were added, mixed and incubated at -70°C for 30 minutes to precipitate DNA. The tubes were then centrifuged for 20-30 minutes in microfuge at 14000rpm. The supernatant was then discarded and the pellet was washed with 70% cold ethanol and centrifuged for 10 minutes at 14000rpm. The supernatant was again discarded and the pellet was allowed to dry for 5 minutes in a speedvac and resuspended in 50 μl TE or distilled water to dissolve.

2.5.8.4. Agarose Gel Electrophoresis of cDNA Probe

The 50µl of DNA was then mixed with 6 µl 40% sucrose and pipetted into the wells of the 1% low melting point agarose gel. The DNA was electrophoresed in 1 x TAE buffer at a constant voltage of 60V for 45 minutes. An appropriate molecular weight marker was run to be able to track the migration of the bands in the gel. The separated bands were viewed under the UV transilluminator and the appropriate bands were cut out with a sterile blade, cutting as little of the agarose around the band as possible. The excised bands were then weighed and dropped into a 1.5 ml Eppendorf.

2.5.8.5. Qiaex II gel extraction kit

The bands were then purified with the aid of the Qiaex II Gel Extraction Kit. A volume of 0.3 ml QX1 solubilization buffer was added per 100 mg of gel. After solubilization 10 µl of Qiaex beads was added to the Eppendorf and the suspension was then incubated at 50°C for 10 minutes, with vortexing at 2 minute intervals. The samples were then centrifuged in a microfuge for 30 seconds. The supernatant was then carefully removed and the pellet was then washed in 0.5 ml QX1 buffer to get rid of residual agarose. The pellet was then resuspended in 0.5 ml equilibration buffer PE and washed twice to the remove the salt contaminants. The pellet was then left to air dry for 15 minutes until it became white. The DNA was eluted with the addition of 20 µl TE buffer to the pellet, which was then vortexed, incubated at room temperature for 5 minutes and then centrifuged for 30 seconds. The supernatant, now containing the DNA, was carefully removed. This elution process was repeated twice. The purity and concentration of the DNA was determined through spectrophotometric readings and 0.8% agarose gel electrophoresis.

2.5.8.6. Labelling of cDNA probe

The Expand ^{TM} Long Template PCR System was used (Boehringer Mannheim, Cat No. 1681 842). This kit contained non-radioactive components to be used with radiolabelled dATP. Unlabelled dCTP, dGTP, dTTP (supplied with the kit) and then 1µl [α ^{32}P] dATP were the nucleotides incorporated during DNA

synthesis, achieving efficient incorporation of the label. Two of the OTC primers were used to amplify the cDNA. These primers were OTCEx1Forward and OTCEx10Reverse at a concentration of 20 pmoles/µl.

2.5.8.7. PCR Amplification Protocol.

All the reagents were thawed and kept on ice. Both primers amplified at a final $MgCl_2$ of 1.5mM and an annealing temperature of 55 ° C or 52 °C.

Reaction Mix per Sample.

4.0 µl	10X PCR Buffer
3.5 µl	dNTP's (C, T, and G) (5 mM)
1.0 µl	OTCEx1Forward (20 pmoles/µl)
1.0 µl	OTCEx10Reverse (20 pmoles/µl)
26.5 µl	ddH_20
1.5 µl	$MgCl_2$ (1.5 mM)
3.0 µl	[α ^{32}P] dATP
---	
41 µl	

Enzyme Mix per sample

0.5 µl	Enzyme (kit)
0.5 µl	10X Buffer
4.0 µl	ddH_20

5.0 µl	

4.0 µl Template DNA (OTCcDNA-0.05 ng/µl)

2.5.8.8. Thermocycling

Primers, OTC Exon1 Forward and OTC Exon10 Reverse, amplified at an annealing temperature of 55°C.

Step 1. Denaturation at 95°C for 3 minutes

2. "hot start" at 75°C for 1 minute

This was set for 1 cycle.

3. Denaturation at 95°C for 30 seconds
4. Annealing at 55°C for 1 minute
5. Extension at 72°C for 1 minute

This was repeated for 10 cycles.

6. Denaturation at 92°C for 30 seconds
7. Annealing at 55°C for 1 minute
8. Extension at 72°C for 1 minute

This was repeated for 23 cycles.

9. Final extension at 72°C for 5 minutes.

2.5.8.9. Hybridisation

All incubations took place in a rolling hybridisation cylinder in a hybridisation oven. The membrane with the transferred DNA on it was rolled in a piece of nylon mesh, which was cut to approximately the same size as the membrane. This was then placed in the hybridisation cylinder with 60ml hybridisation buffer and incubated at 64°C for approximately 2 hours. Heating it at 95°C for 10 minutes denatured the Probe. The hybridisation buffer was poured off and replaced with an equal volume of prewarmed Hybridisation buffer, which had the probe, added to it. This was incubated at 64°C overnight. Note that preheating of the hybridisation buffer is critical for uniform blocking of the membrane, as failure to preheat the buffer results in absorption of the DNA probe by the blocking agents. Also note that the concentrated probe should not be poured directly onto the membrane as localised background may occur. The probe was poured off and stored at –20°C for reuse. The membrane was placed in a glass dish and washed with 250 ml 2XSSC/0.1%SDS for 10 minutes at room temperature, changing the solution after 5 minutes. Then the membrane was washed with 0.2XSSC/0.1%SDS for 10 minutes at room temperature, changing the solution after 5 minutes. After the washes, the membrane was wrapped in Saran Wrap and checked for excessive radioactivity with the Geiger counter, to ensure that the washes had been sufficient.

2.5.8.10. Autoradiography

The membrane was wrapped in Saran Wrap and placed in an X-ray film cassette, covered with Hyperfilm MP (Amersham), and exposed at –70°C for 7 days.

Materials and Reagents: Developer solution

Stop solution (2% acetic acid)

Fixer solution

A 1:3 dilution was made and the pH and silver content was checked.

2.5.8.11. Protocol for developing of the autoradiograph

In the darkroom (using the red light only) the cassette was opened, the autorad removed and placed in developer solution for 90 seconds, in stop solution for 30 seconds and in the fixer solution for 2 minutes. The autoradiograph was then placed in running tap water for 5 minutes and left to dry.

Chapter 2.6: Results

SSCP analysis of PCR amplified coding sequences.

Kindred 1, Patient 1

SSCP analysis revealed an abnormal migration pattern of ssDNA fragments from exon 4 of patient 1 (fig. 12).

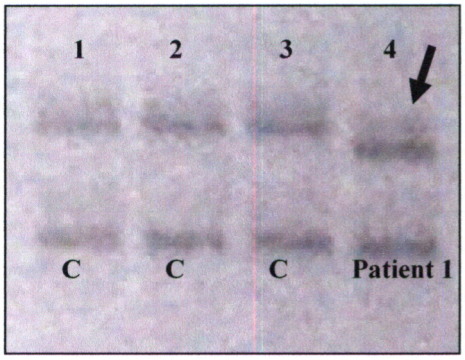


Figure 12. An SSCP gel showing the banding pattern of the single strands from the PCR product of exon 4. The bands from patient 1 are depicted on the right (lane 4) and preceded by the bands from 3 control individuals (lanes 1-3).

Exon 4 was sequenced and an A to T transversion found at nucleotide position 438 of the OTC cDNA (Accession number D00224). This substitution occurred in codon 101 of the mature OTC protein and resulted in substitution of phenylalanine for leucine. This transversion converts the TTA codon for leucine to TTT, which codes for phenylalanine (L101F). The sequencing trace and summary details are given in figures 13 and 14 respectively. Substitutions at the 3rd position of codons are usually inconsequential, as this position is degenerate. However, several amino acids are determined by the nature of the base in this position; phenylalanine and leucine being two of these amino acids. Currently there are 13 different mutations reported in exon 4 in the OTC database. L101F appears in the database and is reported as a polymorphism.

Another single base substitution was found in codon 104 of the mature OTC protein, which results in substitution of glutamine for leucine at nucleotide position 446. This transversion converts the CTG codon for leucine to CAG,

which codes for glutamine (L104Q). The sequencing trace and summary details are likewise given in figures 13 and 14 respectively. L104Q appears to be a novel mutation, as it is not listed in the database.

Subsequently, maternal DNA from the mother and two female siblings of patient 1 was amplified and sequenced to screen for the L104Q mutation and L101F polymorphism. The results revealed that the mother is a carrier for she is heterozygous for the L104Q mutation. One of the female siblings was also found to be heterozygous for the L104Q mutation, making her a carrier as well. The other sibling is homozygous for the normal sequence. Both female siblings and the mother tested positive for the L101F polymorphism. The sequence traces showing the base changes mentioned above are given in figure 15.

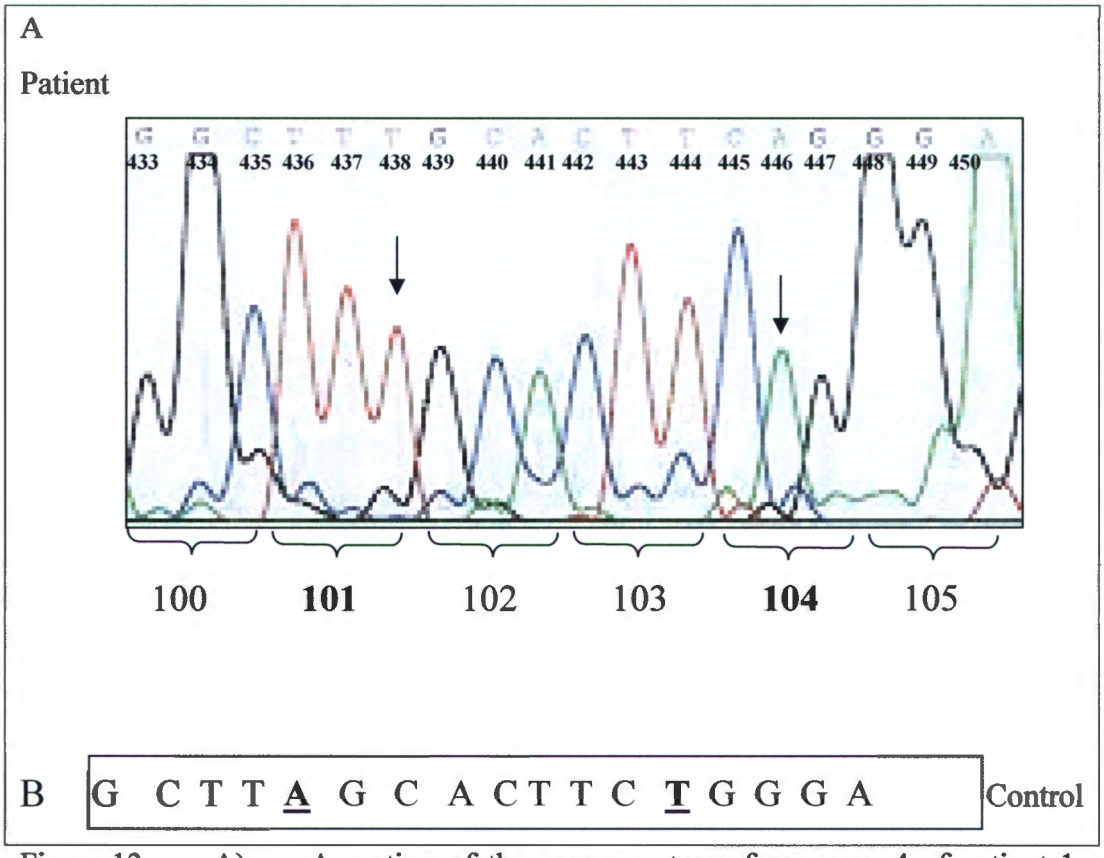


Figure 13. A) A section of the sequence trace from exon 4 of patient 1, spanning codons 100 to 105 and showing the two transversions. B) A section of the sequence trace from exon 4 of a control spanning codons 100 to 105.

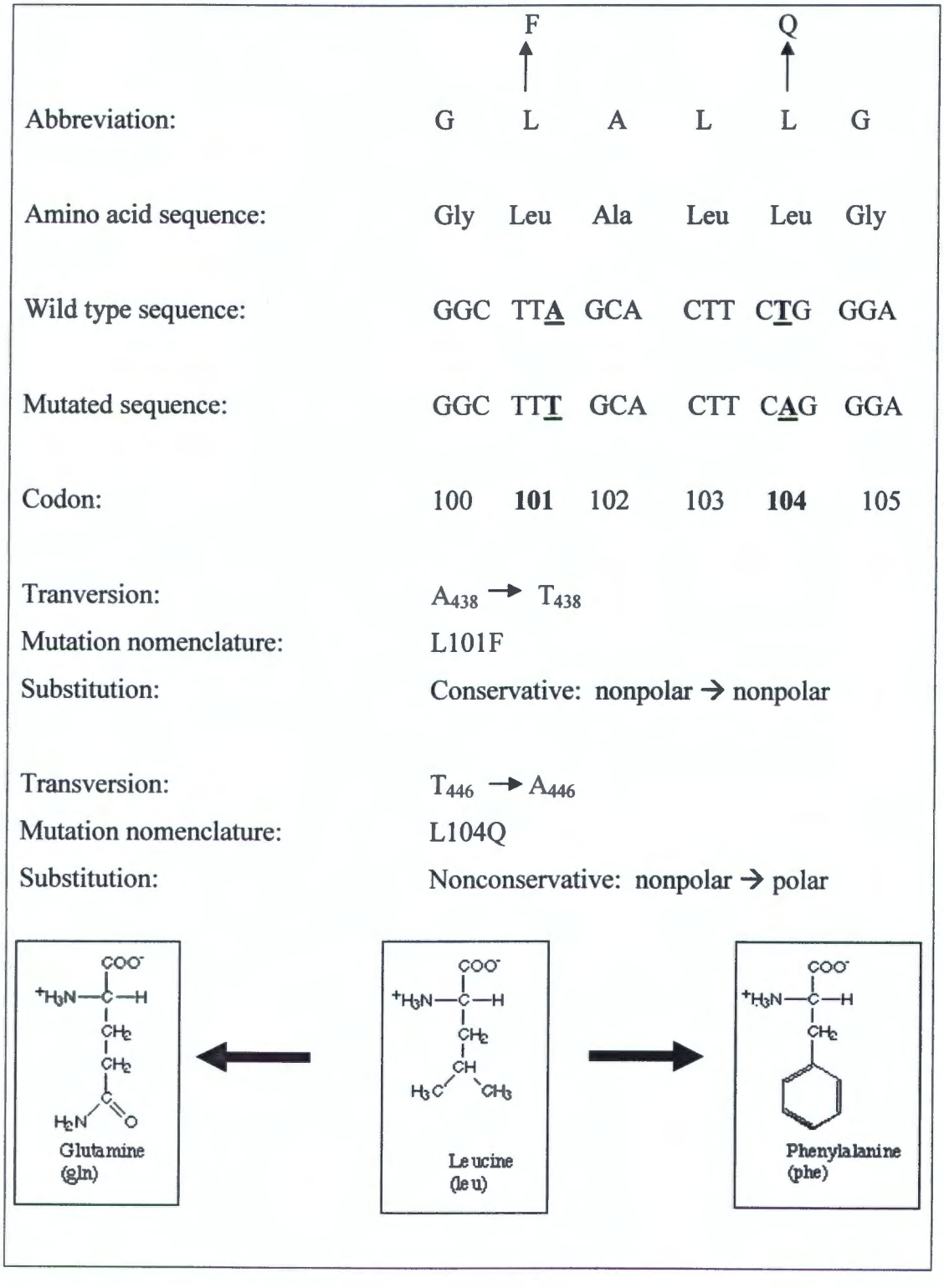


Figure 14. A composite of the characteristics and nature of the polymorphism and mutation in exon 4 of the OTC gene of patient 1. Numbering is taken from the DNA sequence lodged in Genbank under accession number D00224.

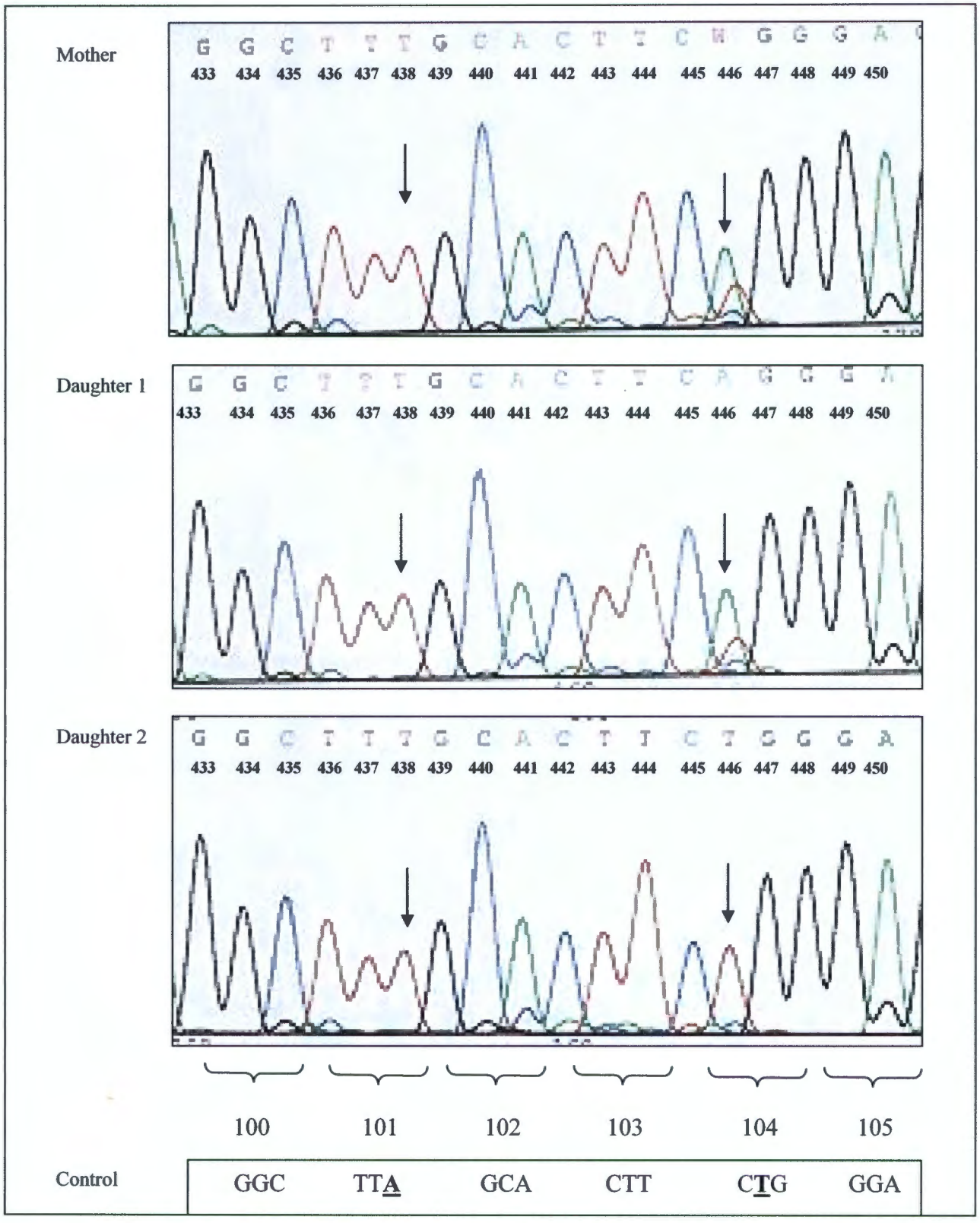


Figure 15. Kindred 1: Sequence traces of the mother (carrier) and her two daughters (Daughter 1 – carrier and daughter 2 – normal) showing homozygosity for the T polymorphism at nucleotide 438 / codon 101 in all three traces in addition to heterozygosity for the A/T mutation at nucleotide 446 / codon 104 in the mother and daughter 1.

Table 1 Sequence conservation of OTC Exon 4 amongst species

Species	Sequence
Amino Acid (homo sapiens) **Codons**	Gly leu ala leu leu gly gly his pro **100 101 102 103 104 105 106 107 108**
Homo Sapiens (humans)	GGC TTA GCA CTT **CTG** GGA GGA CAT CCT
Mus musculus (house mouse)	GGC TTT GCT CTG **CTG** GGA GGA CAC CCT
Bos taurus (cow)	GGC TTT GCA CTT **CTG** GGA GGA CAC CCT
Rattus norvegicus (Norway rat)	GGC TTC GCT CTT **CTG** GGA GGA CAT CCT
Rana catesbeiana (bullfrog)	GGA TTT GCT TTA **TTG** GGA GGA CAT CCT
Trachemys scripta elegans	GGG TTT GCT CTC **CTT** GGG GGA CAT CCT
Gallus gallus (chicken)	GGA TTT GCT CTC **CTT** GGA GGA CAT TCT *
Sus scrofa (pig)	GGC TTT GCC CTT **CTA** GGA GGA CAC CCT

* Pro ⟶ Ser

Other mutations have been reported in close proximity i.e. at codons 100 (gly to asp), 102 (ala to gly) and 106 (gly to arg) suggesting that amino acid sequence conservation in this section of the polypeptide chain is critical for enzyme function. This is also borne out by the conservation of sequence in different species (table 1). Only one amino acid change is found in the 9 amino acid segment shown in table 1, this being a substitution of proline by serine in chicken.

Kindred 2, Patient 2

SSCP analysis revealed an abnormal migration pattern of ssDNA fragments from exon 2 of patient 2 (Fig 16).

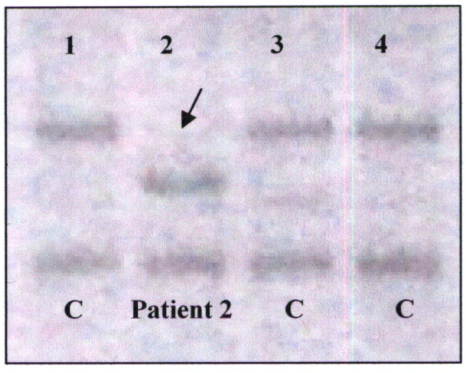


Figure 16. An SSCP gel showing the banding pattern of the single strands from the PCR product of exon 2. The bands from patient 2 are shown in lane 2 and surrounded by the bands from three control females (lanes 1, 3 and 4).

Exon 2 was the only exon that yielded a band shift on SSCP analysis and was therefore sequenced to determine the base alteration. This proved to be an A to G transition at nucleotide position 271 which occurred in codon 46 and resulted in a substitution of arginine for lysine (K46R). The sequencing trace and summary details are given in figures 17 and 18 respectively. This mutation is not novel as it is currently listed as a polymorphism in the OTC mutation database. Polymorphisms are sequence alterations in the DNA that have no discernable impact on gene expression or gene product function. They also appear at frequencies of > 1% in the general population. Mutations on the other hand are DNA sequence alterations that result in altered function of gene products, either on the basis of abnormal gene expression or product function.

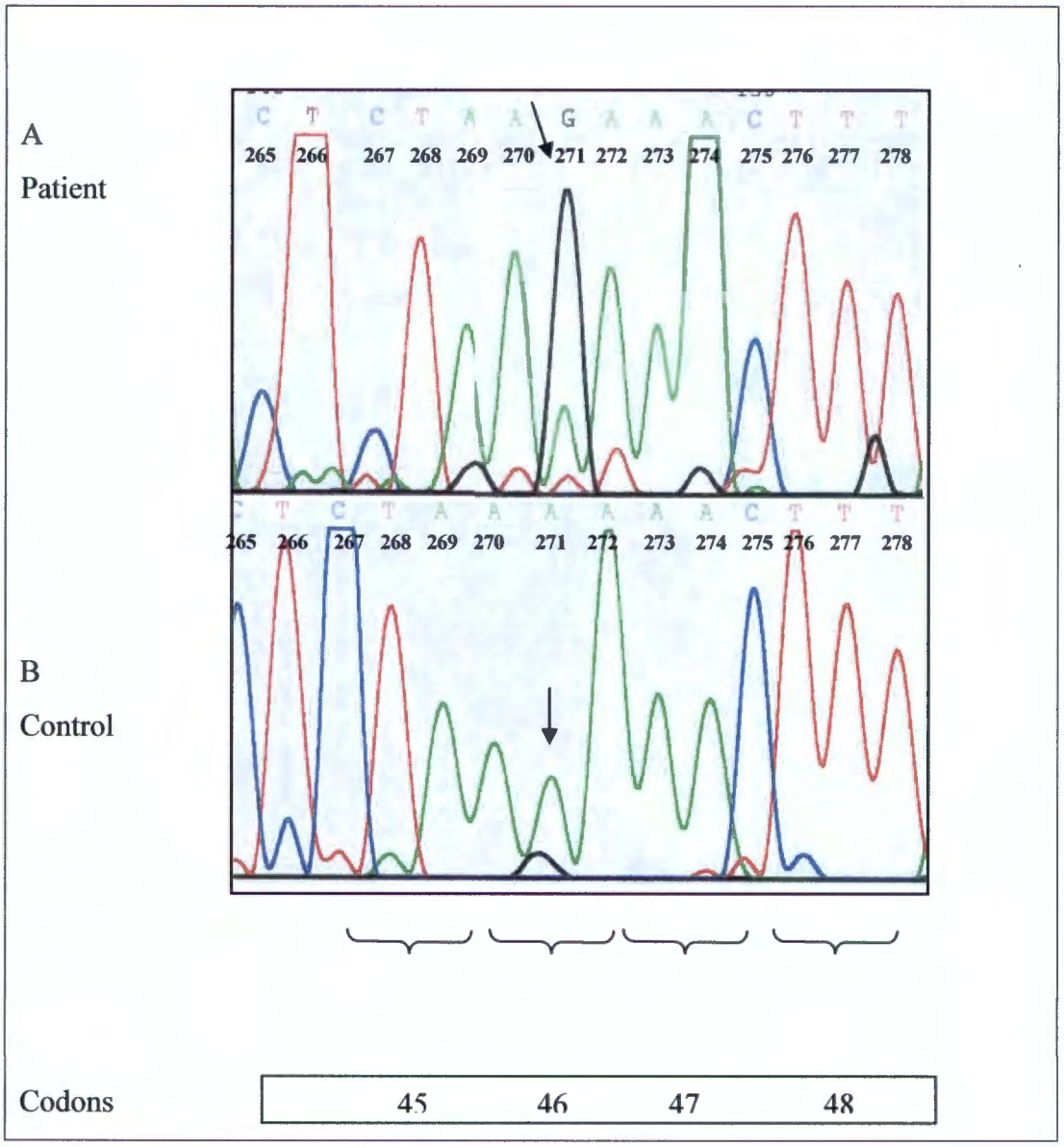


Figure 17. Aligned sections of the sequencing traces spanning codons 44 to 48 of exon 2 of the proband (A) and a control female (B).

Complete exon sequencing

It is common knowledge that SSCP coverage of sequence alterations is not 100% effective. It was therefore decided to sequence the remaining OTC exons from patient 2. These were duly carried out with each exon yielding normal coding and splice site sequences.

			R ↑		
Abbreviation:	T	L	**K**	N	F
Amino acid sequence:	Thr	Leu	Lys	Asn	Phe
Wild type sequence:	ACT	CTA	A**A**A	AAC	TTT
Mutated sequence:	ACT	CTA	A**G**A	AAC	TTT
Codon:	44	45	46	47	48
Transition	$A_{271} \rightarrow G_{271}$				
Mutation nomenclature:	K46R				
Substitution:	Conservative: both basic residues				

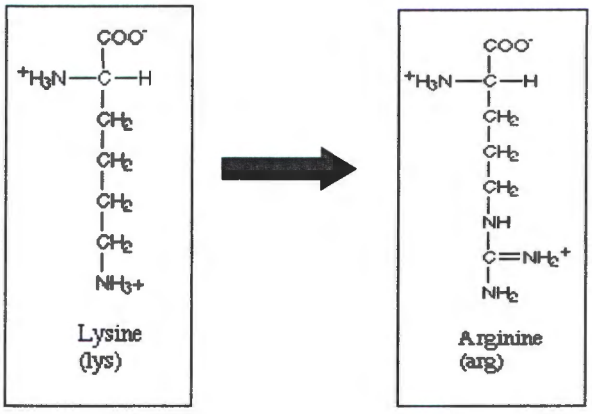


Figure 18. A composite of the characteristics and nature of the polymorphism in exon 2 of the OTC gene of patient 2. Numbering is taken from the DNA sequence lodged in Genbank under accession number D00222.

Verification of the K46R polymorphism

The A-to-G substitution at codon 46 of exon 2 in patient 2, created a cutting site for the restriction endonuclease *DdeI* which has the following recognition sequence: C ↓TNA G (Fig. 19A). The location of the cutting site within the exon is shown in fig. 19B. Digestion of the PCR product from the mutant allele generates two fragments of sizes 126bp and 118bp which can be separated on a 20% PAGE gel as shown in fig. 20. This polymorphism is reported as being a low frequency one, as it occurs in less than 10% of the population. (Grompe, 1989) Interestingly, two of the six controls in our study also showed this polymorphism. (Fig. 20 lane 8). Screening of further controls to determine the frequency of this polymorphism in the South African population was not carried out.

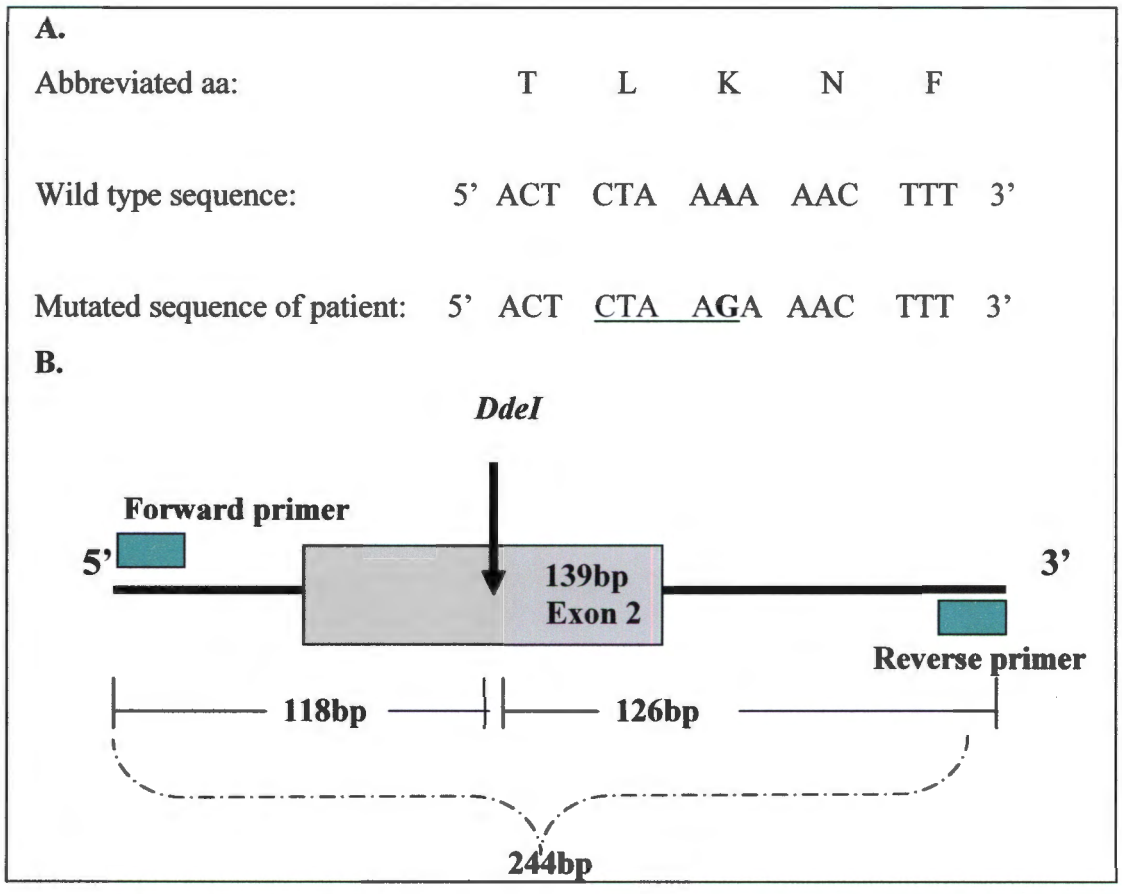


Figure 19. A) The nucleotides in bold indicate the normal and mutated bases respectively. The underlined region represents the *DdeI* cutting site. B) A schematic presentation of exon 2 showing the position of the new *DdeI* site and the sizes of the exon 2 fragments generated by restriction of the PCR product.

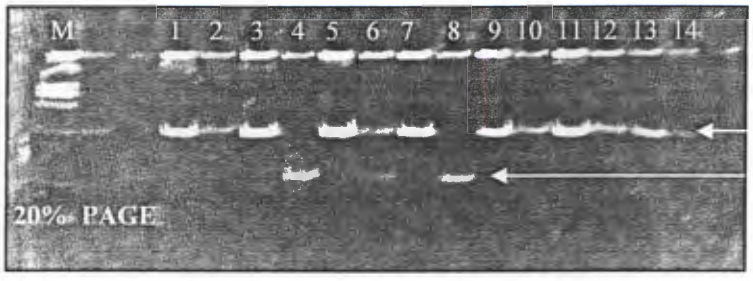


Figure 20. Kindred 2: Restriction analysis of exon 2 with DdeI. Lane M: Molecular weight marker VIII, Lane 3: undigested PCR product of patient 2 (244bp), Lane 4: digested PCR product of patient 2 (126bp and 118bp), Lanes 1, 5, 7, 9, 11 &13: undigested amplified DNA (244bp) of controls, and Lanes 2, 6, 8, 10, 12 & 14: digested PCR products of male controls. Controls in lane 6 and 8 had the common polymorphism. The 118bp band has run off and is not visible on this gel.

Kindred 3, Patient 3

PCR amplification of the OTC exons from patient 3 was successful for exons 1-4 but not for exons 5, 6, 7, 8, 9, and 10 (Fig 21). These data are indicative of a partial deletion of the OTC gene, which incorporates exons 5-10 with one of the breakpoints occurring in intron 4. This mutation is novel as it is not listed in the OTC mutation database. Currently 5 large deletion mutations are recorded with none of them having a breakpoint in intron 4.

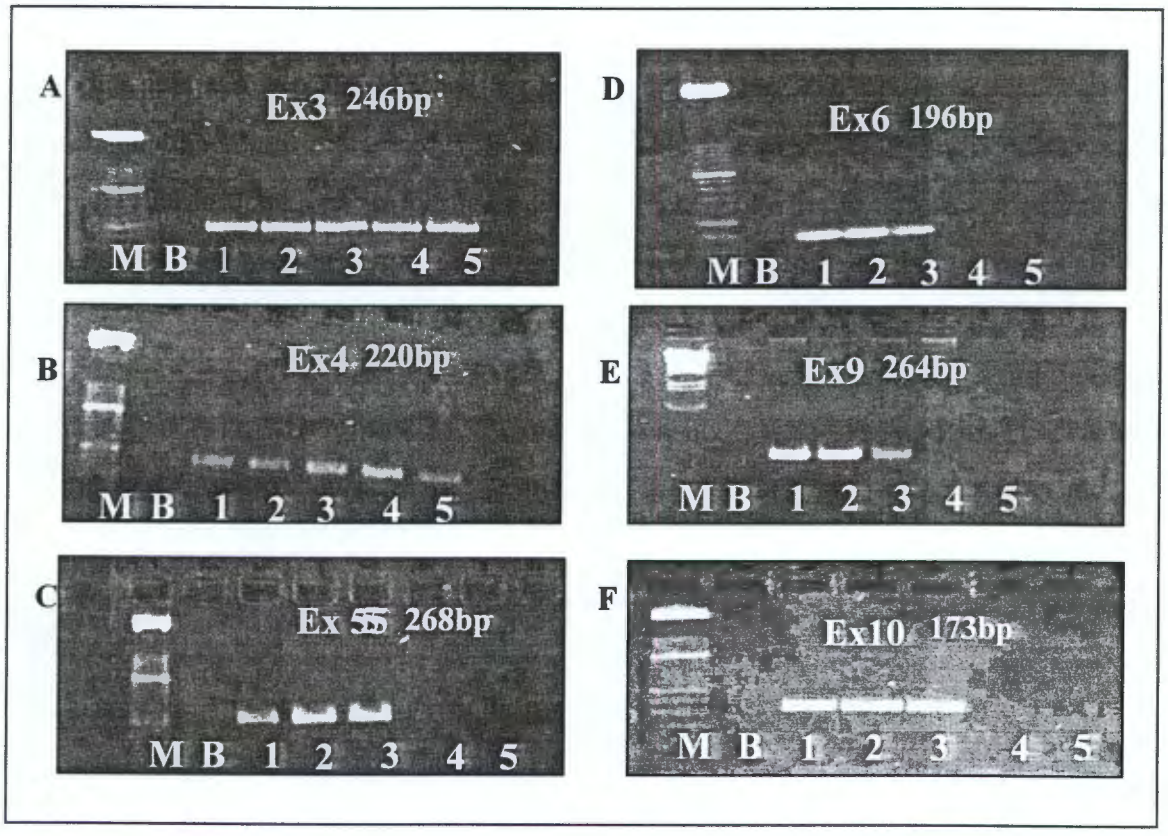


Figure 21. A catalogue of some of the post-electrophoresis agarose gels, which show the successful amplification of exons 3 – 4 but not exons 5 – 10 from DNA of patient 3. M = DNA marker, B = Blank, 1 = PCR product from control DNA, 2 =PCR product from maternal DNA, 3 =unaffected brother, 4 =previously affected brother, and 5 =patient 3.

RFLP analysis and Southern Blotting

Confirmation of the deletion mutation in patient 3, via the Southern Blotting (SB) technique was attempted but eventually discontinued due to major delay in probing the blot caused by the inadvertent use of a non OTC cDNA probe. Time constraints on the completion of the thesis did not allow for further southern blotting attempts. The basics of the SB method have however, been included in addition to the picture of the pretransfer gel as a significant period of time was expended on this method. The major delay was caused by an overseas laboratory, which supposedly sent us three OTC cDNA clones, which we eventually discovered were not OTC cDNA clones as the restriction patterns of several restriction enzymes differed from that predicted from the OTC cDNA sequence in Genbank. The clones received were labelled as DH5α pUC19 OTC, pHOE1, and pETOTCM2. The clone first utilised was pUC19 OTC with the insert cloned into the Pst1 site of the plasmid. Digestion with *Pst1* gave an insert of the expected size of the OTC cDNA. This band was excised and purified, using a Qiagen Kit. PCR amplification was attempted using the OTC primers, which were supplied with the clone (pZ106 and pZ107), but was unsuccessful. These primers were thought to be the wrong primers. Numerous attempts at optimisation were made but without success. It was then decided to check the identity of the cDNA insert by restriction digestion.

The inserts from the other two clones, pHOE1 and pETOTCM2 were also digested with various restriction enzymes and again the fragment patterns obtained differed from those expected from the OTC cDNA. By the time we received the correct OTC cDNA probe (Figures 23 and 24) from a different laboratory, my initial blot did not work and there was insufficient time available to repeat the blotting and probing procedures.

Restriction endonuclease digestion

Before transfer, the digested electrophoresed DNA samples were photographed in the gel under UV light, allowing for calculation of molecular weights of bands to be observed on the autoradiograph (fig. 22.).

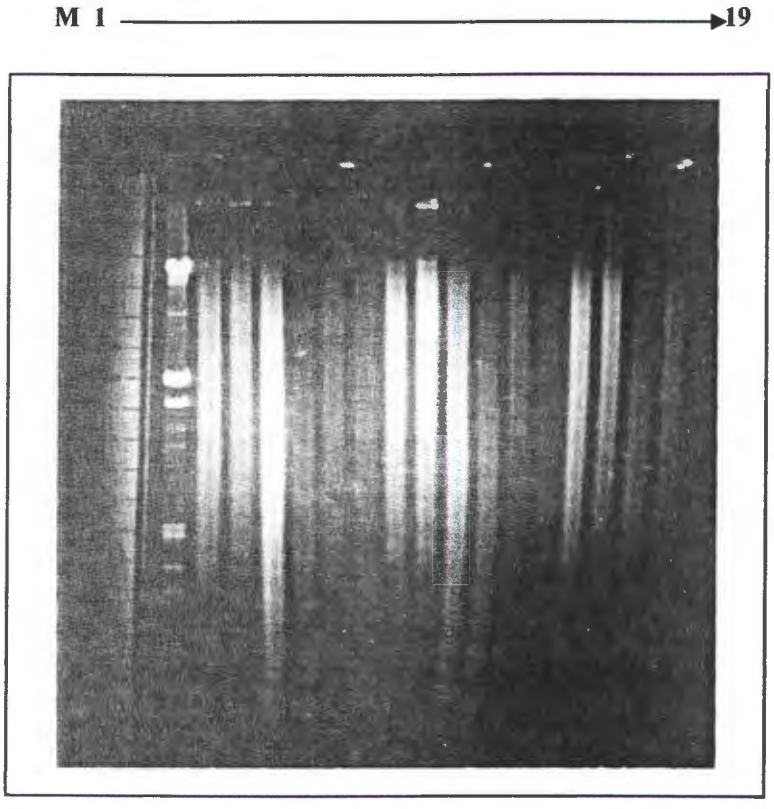


Figure 22. Electrophoresed genomic DNA samples digested with various restriction enzymes, prior to Southern blotting. The DNA samples were digested with enzymes, *Pst1, HindIII and EcoR1*, and loaded onto the gel as follows: Lane 1, Molecular weight marker *HindIII / EcoR1*; lane 2, control 1; lane 3, control 2; lane 4, mother of patient 3; lane 5, normal son; lane 6, previously affected son; lane 7, patient 3; lane 8, control 1; lane 9, control 2; lane 10, mother of patient 3; lane 11, normal son; lane 12, previously affected son; lane 13, patient 3; lane 14, control 1; lane 15, control 2; lane 16, mother of patient 3; lane 17, normal son; lane 18, previously affected son; and lane 19, patient 3.

Generation of an OTC cDNA probe

An authentic OTC cDNA probe was eventually prepared from clone pH0731, kindly supplied by Dr. Wayne Fenton, Yale University. The full length OTC cDNA has a translatable size of 1065bp. Primers flanking the coding sequence were selected from the 5' and 3' non-translated regions of the OTC cDNA as shown in Fig. 23. These primers gave a cDNA probe of 1159bp in length (Fig 24), which included all ten exons of the OTC gene. This fragment was labelled by nick translation, gel purified and used as a probe in the single hybridisation attempt.

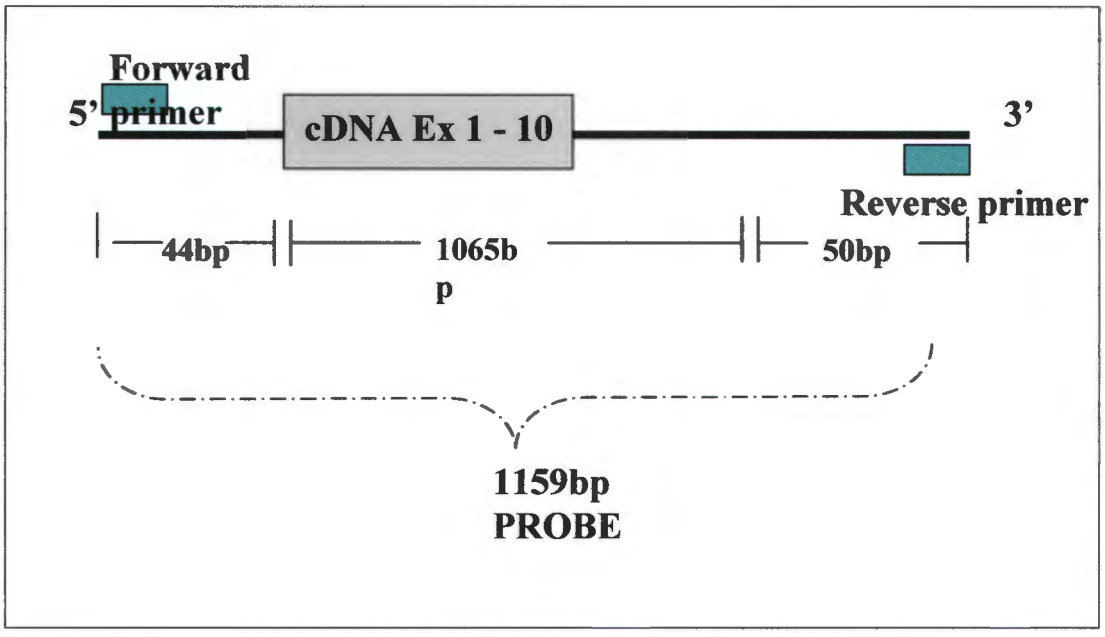


Figure 23. Diagram of the OTC cDNA sequence of clone pH0731, showing the location of the forward and reverse primers as well as the exonic sequence.

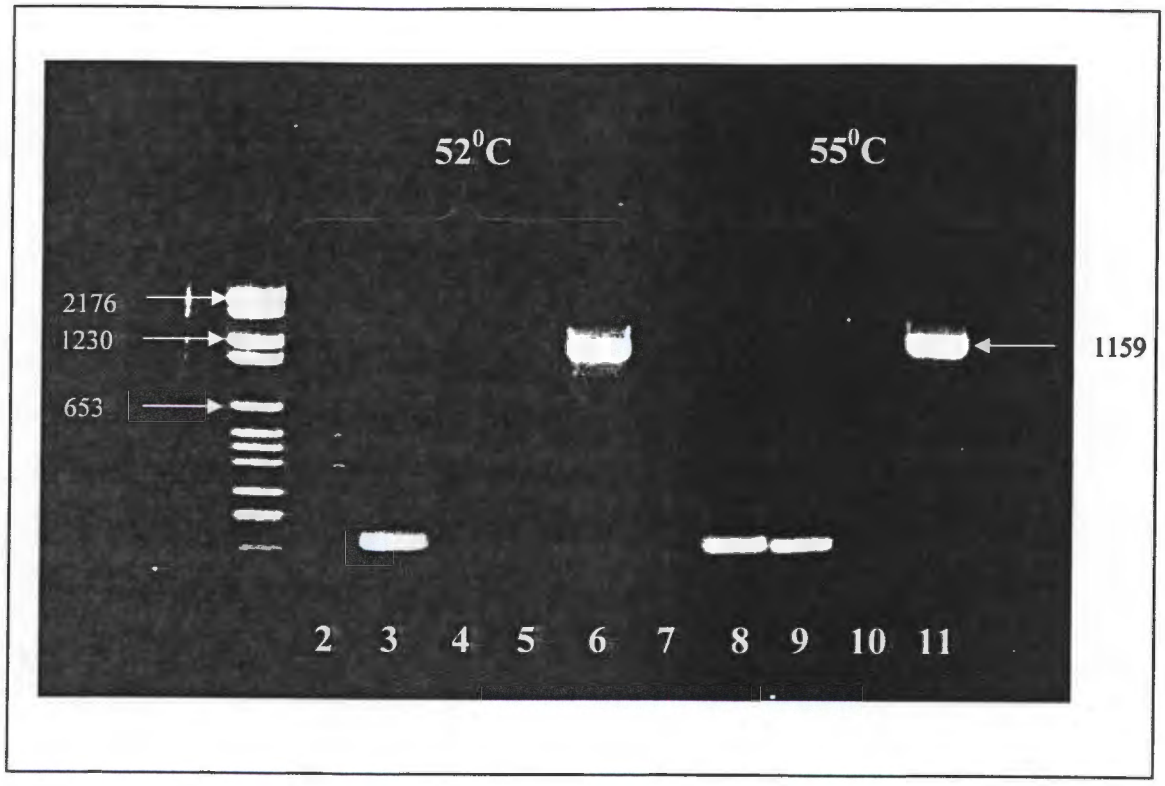


Figure 24. Polyacrylamide gel electrophoresis of the 1159bp PCR product amplified for the OTC cDNA (pH0731), which was used the hybridisation probe. Lane 1: Molecular weight marker VI; Lane 2, PCR blank; Lane 3, Control DNA exon 10; Lane 6, PCR product at annealing temp of 52°C; Lanes 8 & 9, Control DNA exon 1 and 10 respectively; Lane 11, PCR product at annealing temp of 55°C. Both annealing temperatures worked well for the amplification of the cDNA. Exons 1 and 10 were amplified in the same run to demonstrate primer amplification efficiency, as the respective forward and reverse primers were used to amplify the cDNA fragment of clone pH0731.

Time constraints prevented further characterisation of the deletion through Southern blotting.

2.7. Discussion and Conclusion

OTC deficiency is typical of other X-linked conditions where gene mutations are predominantly family specific or private, with only a few occurring recurrently and these at a frequency generally less than 5%. Although this absence of founder or population specific mutations complicates genetic diagnoses considerably, the effort involved is worthwhile as mutation identification provides accurate diagnosis, a reliable tool for carrier testing and prenatal diagnosis for which alternative biochemical and enzymatic methods are less reliable or invasive.

This chapter has detailed the mutation workup in three kindreds with children affected by OTC deficiency. The mutation found in Kindred 1 was shown to be a T → A transversion in codon 104 of exon 4. This transversion converts the CTG (leucine) codon to CAG that codes for glutamine. This mutation (L104Q) is novel, as it is not listed in the OTC mutation database. Also in the same exon of this patient we identified a known polymorphism in codon 101. This polymorphism (transversion) converts the TTA codon for leucine to TTT, which codes for phenylalanine (L101F). Two findings suggest that L104Q is a mutation and not a polymorphism. Firstly, the amino acid substitution is nonconservative, in that nonpolar leucine has been substituted by the polar glutamine. Secondly, other mutations have been reported in close proximity i.e. at codons 100, 102 and 106, indicating that this section of the polypeptide chain is critical for enzyme function. It has also been shown that this specific region in exon 4 is highly conserved (table 1), which further strengthens the fact that this finding is a novel mutation. A stronger case could have been made if the L104Q substitution was shown to be absent in a large number of chromosomes from control subjects. Ultimate verification however, must await mutagenesis, transfection studies and enzyme activity analysis

While this study successfully identified a known polymorphism in the OTC gene in patient 2, it was unable to confirm the diagnosis of OTC deficiency through mutation detection. DNA from patient 2 was additionally subjected to PCR sequencing for each of the 10 exons of the OTC gene, but still without success.

Further gene studies in family 2 were not carried out as there is a strong possibility that the proband falls into the 20-30% of OTC deficiency patients who manifest with a normal OTC coding and splice site sequence and presumably carry mutations in the promoter or intronic regions of the OTC gene.

The mutation in kindred 3 was shown to be a large intragenic deletion removing exons 5 – 10. The size of this deletion is unknown but it was shown, through the use of OTC microsatellites (see chapter 4) to be less than 1 cM as two of the flanking OTC markers (DXS8090 and DXS8113) could be amplified; these two markers are approximately 1 cM apart. This null mutation is likely to generate an unstable partial OTC mRNA as it lacks exon 10 and the 3' untranslated region, which contains the polyadenalation signal. If translation does occur then the truncated protein produced is likely to be rapidly degraded.

The failure of mutation detection in Kindred 2 has implications for prenatal diagnosis in that male foetuses conceived in the future, will have to be screened using microsatellite markers for the OTC gene. This microsatellite approach was in fact used for Kindred 3 where prenatal diagnosis was requested in two pregnancies before the mutation in this family had been characterised (refer to chapter 4). The choice for microsatellite usage in kindred 2 was given by the strong history of X-linked inheritance, through the multiple early male deaths, and the clinical and laboratory presentation of OTC deficiency in the proband.

Chapter 3.1: Mutation analysis and X-inactivation studies: Kindred 4

Overview

This kindred has been dealt with in a separate chapter as the proband, patient 4, while presenting with the entire hallmark features of late onset OTC deficiency was also found to have Klinefelter's syndrome. Considerable time has been spent in trying to substantiate the presence of OTC deficiency in the face of Klinefelter's syndrome where the patient now has two copies of the X chromosome.

Patient 4

Patient 4 was born in 1996 and is part of a singleton family (Fig 25). His neonatal period was relatively uneventful and he remained well until the age of 18 months when his health deteriorated and he started having episodes of vomiting and associated periods of ataxia and intentional tremor.

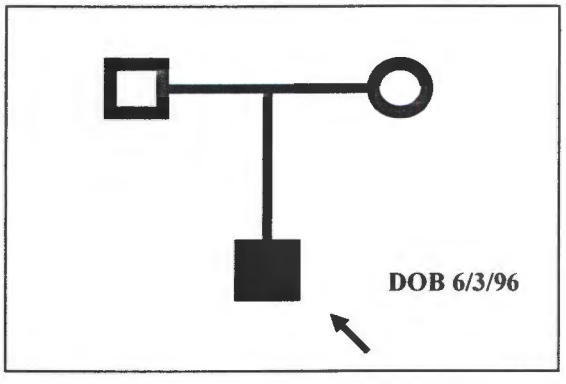


Figure 25. The family tree of patient 4.

He was referred to Red Cross Children's Hospital at this time and was diagnosed with hepatitis and subsequently sent home. Shortly afterwards, he presented with self-mutilating behaviour, recurrent episodes of vomiting and lethargy, ataxia, and seizures. Blood ammonia was recorded at 600 μMols/L (normal <50). These symptoms were suggestive of a late onset urea cycle defect with OTC deficiency

being the most likely candidate. Plasma amino acid levels were found to be normal as was the citrulline level. Urine orotic acid excretion was found to be more than three times normal at 11.1 nM/L (normal = <3). The clinical and laboratory presentation was compatible with OTC deficiency, which is the most common of the urea cycle defects.

DNA studies

The diagnosis of OTC deficiency is usually based on a liver biopsy and assay of OTC activity. This option however, was not available for this patient and it was decided to screen genomic DNA for mutations in the OTC gene. PCR followed by SSCP was performed on all the exons and no evidence was found for any exonic mutations.

The next step in the workup in this patient would have been the sequencing of each of the 10 exons of the OTC gene, as it is known that mutation detection via the SSCP technique is not 100% effective. However, during the SSCP studies it was discovered that DNA from patient 4 had earlier been sent to an overseas laboratory for sequencing. This sequencing exercise had been terminated when preliminary results indicated the presence of two copies of the X-chromosome. Patient 4 had then been karyotyped and were found to have Klinefelter's syndrome (47XXY).

Given this new information, it was decided to expand the range of methodologies normally employed in mutation detection studies, by determining whether skewed X-inactivation accounted for the combination of OTC deficiency and Klinefelter's in our patient. This investigation would also determine whether two copies of the mutant X-chromosome had been inherited from his mother. Comprehensive sequencing of all the OTC exons was to be undertaken after this investigation.

X-inactivation

Background: Males have one X chromosome, while females have two. This creates a dosage imbalance for X linked genes, which is addressed by the cell through the inactivation of one of the X chromosomes, leading to an active and an inactive

chromosome. The latter X chromosome can be distinguished by the fact that it is late replicating, methylated at the gene control regions, transcriptionally repressed and heterochromatic. Inactivation occurs during at the blastocyte stage of development is random. Females therefore who are heterozygous for X-linked traits should have half their cells expressing either of the X chromosomes. In reality, 5 to 10% of normal females demonstrate non-random inactivation and are said to be skewed for X inactivation. In this scenario, the cell type expressing one X will predominate over the other cell types. So, in females heterozygous for an X-linked disorder, the degree of skewing will correlate with the degree to which they demonstrate symptoms of the disorder. X inactivation therefore plays a key role in the clinical phenotype in female carriers of X-linked diseases. In fact this was first recognized by Lyon in 1961 when he observed that females heterozygous for X-linked genes showed mosaic expression (Willard, H.F, 1995).

X inactivation requires a locus, the X inactivation center (X-IC) on the long arm of the X chromosome for inactivation to occur. The X-IST (X-inactivation specific transcript) gene maps within the X-IC region and plays a role in X inactivation. (Puck, J.M and Willard, H. F, 1998) X-IST is only expressed from the X chromosome selected for inactivation and produces an RNA molecule that spreads an inactivation signal in *cis* up and down the X chromosome while remaining associated with the inactivated X chromosome. (Willard, H.F, 1995).

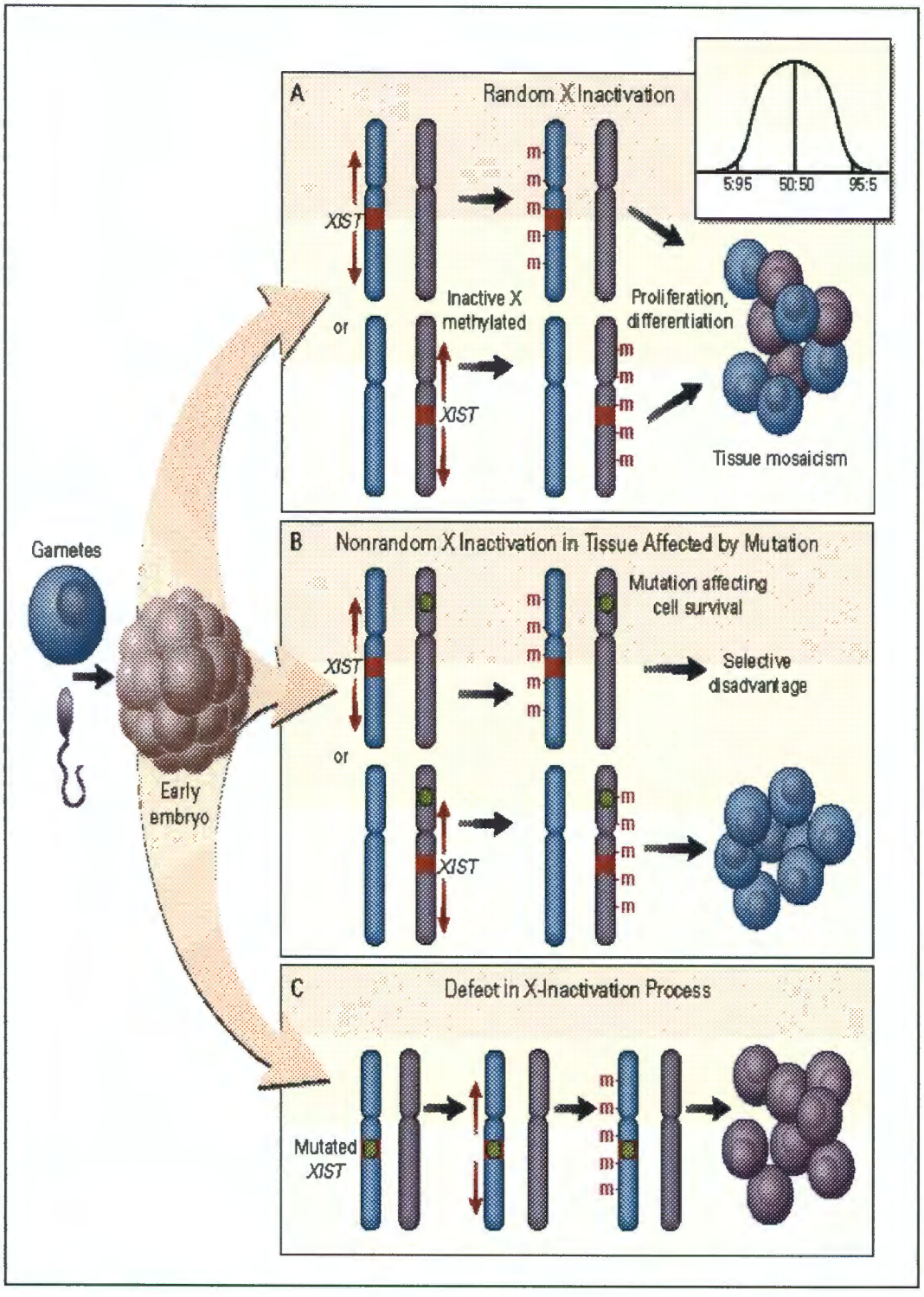


Fig 26. Three mechanisms leading to a Skewed pattern of X chromosome Inactivation. (Puck, J.M and Willard, HF, 1998)

X inactivation is maintained during cellular proliferation and differentiation by continued expression of the X-IST gene and by the methylation of DNA cytosine residues on the inactive X chromosome (Fig 26a). If one X chromosome carries a gene defect that is detrimental to proliferation and/or survival of a particular cell type, then all cells of that lineage will die and the only cells remaining will be those preferentially inactivating the mutated X chromosome (Fig 26b) (Puck, J.M and Willard, H.F, 1998). In Fig 26c, a mutation in the X-IST gene results in the non-random selection of the X chromosome to be inactivated. The red arrow indicates the production of X-IST RNA that spreads an inactivation signal up and down the X chromosome.

Determination of X-inactivation status

The X inactivation pattern in women can be derived from examination of a trinucleotide repeat microsatellite in the first exon of the human androgen-receptor gene (HUMARA) (fig. 27). Two methylation sensitive *HpaII* sites occur less than 100bp away from this microsatellite and have been exploited in a PCR based assay, which can distinguish between the active and inactive X chromosome. This assay utilizes primers, which yield a PCR product of approximately 280 bp that includes the *HpaII* sites and the trinucleotide repeat element.

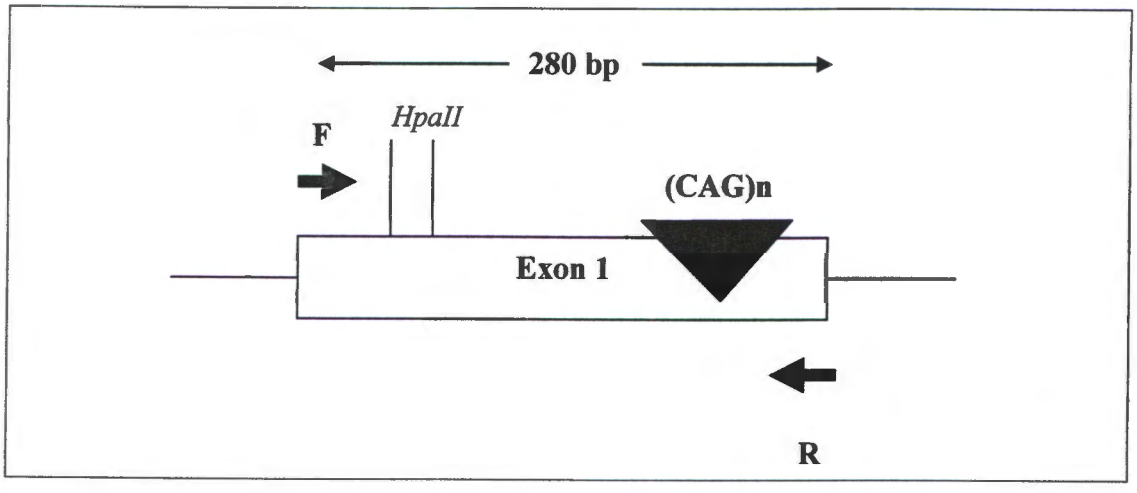


Fig. 27. Exon 1 of the HUMARA locus showing the locations of the Forward (F) and Reverse (R) PCR primers, the CAG repeat and the *HpaII* sites (Wengler G. S. et al, 1997).

When the template DNA is digested, prior to PCR amplification, with *HpaII*, a methyl sensitive restriction enzyme, amplification only occurs from the allele where *HpaII* restriction sites are methylated. This occurs because the template allele containing unmethylated *HpaII* sites is digested and destroyed by *HpaII*. A product will thus only be obtained from the inactivated X chromosome that carries the methylated template (Allen, R. C. et al, 1992).

Because of the highly polymorphic character of the STR most females yield 2 bands of different lengths on electrophoresis. Males with only one X chromosome yield one band when amplified (fig. 28); females yielding 1 PCR band on electrophoresis carry the same length repeat on each of their X chromosomes.

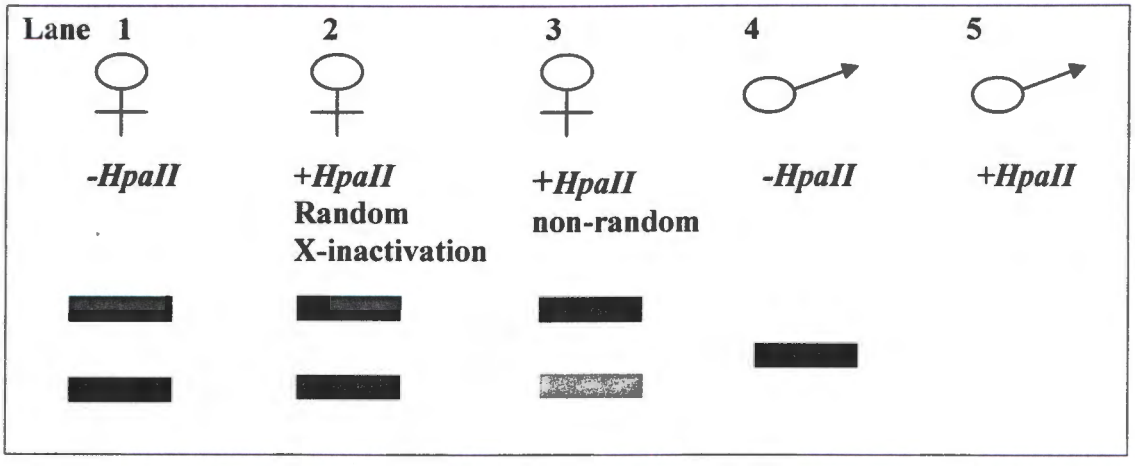


Fig. 28. Graphical representation of the electrophoretic separation of the PCR products derived from the CAG triplet repeat in exon 1 of the HUMARA gene. Lane 1, the two banded pattern from a women, heterozygous for triplet repeat lengths, prior to *HpaII* digestion; lane 2, the banding pattern from the same women post *HpaII* digestion, showing random X-inactivation; lane 3, the banding pattern from a women showing partial skewing of X-inactivation; lanes 4 and 5, the respective band patterns from a man before and after *HpaII* digestion of genomic DNA.

Chapter 3.2: Methods

3.2.1. DNA extraction and quantitation

3.2.1.1. Genomic DNA extraction protocol

3.2.1.1.1. Whole blood extraction method (refer to 2.5.1.1.)

3.2.1.1.2. Frozen blood extraction method (refer to 2.5.1.2.)

The concentration, purity and integrity of the DNA was determined by measuring the OD_{260}/OD_{280} ratio and by viewing the extracted DNA on agarose gel electrophoresis.

3.2.2. DNA quantitation

3.2.2.1. Determination of DNA concentration

3.2.2.1.1. By Spectrophotometric readings A_{260} (refer to 2.5.2.1

3.2.2.1.2. By agarose gel electrophoresis (refer to 2.5.2.2.)

3.2.3. Restriction digestion

3.2.3.1. Protocol for digestion

The following quantities were added to the reaction mixes per 20 µl volume:

10 x Buffer L	2.0 µl
0.4% Casein	2.0 µl
0.04% BSA	0.2 µl
distilled water	9.8 µl
Hpa II (10U/µl) enzyme	2.0 µl
DNA (2µg)	4.0 µl
	20 µl

Samples were incubated @ 37°C overnight. A further 10 U(1 µl) *Hpa II* was added and again the samples digested for another 2 hours. The mixtures were then heat-inactivated @ 95°C for 10 minutes and subjected to PCR amplification.

3.2.4. PCR

3.2.4.1. PCR amplification

The reaction mixtures per 1 sample of 50 µl contained the following volumes:

2.5 mM dNTP mix	4.0 µl
10 x PCR buffer	4.5 µl
25 mM $MgCl_2$	1.0 µl
20 pM ANDR-f primer	1.0 µl
20 pM ANDR-r primer	1.0 µl
distilled water	28.5 µl
digested DNA	4.0 µl
	45 µl

Control samples without DNA were also included. PCR mixtures were overlaid with mineral oil (45 µl) to prevent evaporation and condensation of the samples. Tubes were placed in a thermocycler and subjected to a "hot start" by the addition of the 5 µl enzyme mix after the first 95°C for 5 minutes step in the PCR cycle.

An enzyme mix contained the following quantities per reaction:

Taq polymerase	0.5 µl
10 x Promega buffer	0.5 µl
distilled water	4.0 µl
	5.0 µl

3.2.4.2. PCR thermocycling

The following programme was used:

Step i. Denaturation at 95°C for 5 minutes

This was set for 1 cycle

ii. denaturation at 94°C for 30 seconds

iii. annealing at 58°C for 30 seconds

iv. extension at 72°C for 40 seconds

This was repeated for 28 cycles

v. Final extension at 72°C for 7 minutes

3.2.5. Polyacrylamide gel electrophoresis

3.2.5.1. Preparation of plates

Plates were prepared as in section 2.4.1 and 2.4.2 with a few modifications. Two small plates (150 mm x 150 mm) were used instead of the 2 large (300 mm x 400 mm) plates; 0.75 mm spacers and comb were used instead of the 1mm spacers and comb.

3.2.5.2. Preparation of PAGE gel

The following volumes were added to make the polyacrylamide gel:

20%Acrylamide (19:1)	12.0 ml
5 x TBE	4.8 ml
Urea	4.8 g
10%APS	240 µl
distilled water	7.2 ml
TEMED	15 µl
	~ 24 ml

A well-forming comb was inserted at the top of the two plates and side-clamps were used to hold the plates firmly together. The solution was gently mixed, taken up in a 20 ml syringe and poured between the two plates, which were lying on a flat surface. Polymerisation occurred in about 90 minutes. The gels were then ready for use.

3.2.5.3. Sample preparation and electrophoresis

An aliquot (5 µl) of the PCR product was mixed with 2 µl of the 6 x loading dye. Combs were removed from the gel to form the wells. The gel was placed into the electrophoresis tank and filled with 1 x TBE buffer. Prior to loading, wells were cleaned of urea by rinsing with tank buffer with the aid of a syringe. The gel was run at room temperature @ 65W overnight.

3.2.6. Visualisation of PCR products

3.2.6.1. Silver stain

Following electrophoresis and disassembly the plate containing the gel was placed in a gel tray for silver staining. The gel was covered with 2 litres of distilled water and gently agitated for 1 minute. The water was discarded and Silver Nitrate solution (solution1) added. The tray was slowly agitated for 10-15 minutes, whereupon Solution1 was decanted and replaced by distilled water. After shaking for a further 2 minutes, the water was discarded and solution 2 added to the tray. Agitation was continued for 10-15 minutes or until bands were clearly visible. Solution 2 was discarded and water added to the tray, which was agitated for a further minute. Thereafter the water was discarded and solution 3 was added to the tray to sharpen the bands. Solution 3 was discarded and the gel reimmersed in water. The gel was then viewed and photographed on a light box. The glass plates were soaked in 0.5 M NaOH to remove the gel from the plate. The plates were given a comprehensive rinse and then stored for the next gel.

The exons showing a band shift were then amplified through PCR, purified and submitted for sequence analysis.

3.2.6.2. Ethidium bromide stain

9 µl of EtBr was mixed in 125 ml of 5 x TBE

The gel was gently lifted off the plate and soaked in the EtBr solution (10 mg/ml) for 30 minutes and visualised on the UV transilluminator.

Chapter 3.3: Results,

3.3.1. X-inactivation studies

The microsatellite marker in exon 1 of the Human Androgen Receptor gene was amplified by PCR using both HpaII digested and undigested genomic DNA, which had been isolated from leucocytes of patient 4. The PCR products were run on non-denaturing PAGE gels and were visualised by silver staining (Fig 29). The two faint upper bands in lanes 1 and 2 depict non-specific binding during the amplification step.

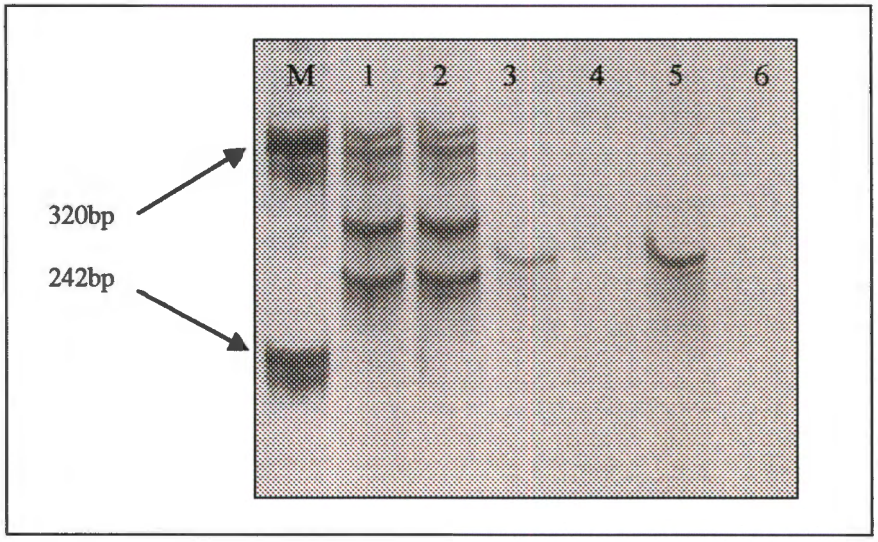


Fig. 29. Polyacrylamide gel electrophoresis of the PCR products from the microsatellite marker in exon 1 of the Androgen Receptor gene. Lane M, Molecular weight marker; lane 1, PCR product of *undigested genomic DNA* of patient 4; lane 2, PCR product of *digested genomic DNA* of patient 4; lane 3, PCR product of *undigested genomic DNA* of male control 1; lane 4, PCR product of *digested genomic DNA* of male control 1; lane 5, PCR product of *undigested genomic DNA* of male control 2, lane 6, PCR product of *digested genomic DNA* of male control 2.

3.3.2. OTC gene sequencing

Each of the 10 exons of the OTC gene in patient 4 have been sequenced and in each case, normal coding and splice site sequences were recorded.

3.4. Discussion and Conclusion

The coexistence of Klinefelter's syndrome with OTC deficiency was a significant complicating factor in the diagnostic workup of patient 4. Carrier females for OTC deficiency can present clinically, and this is usually due to skewed X-inactivation in the liver, where the majority of hepatocytes have inactivated the normal X-chromosome. Skewing of X-inactivation was thus, also a possibility in our patient as Klinefelter's patients always inactivate one of the X-chromosomes in all their cells. There was however a second possibility in our patient and that was the inheritance of two copies of the mutant X-chromosome. Both these possibilities were explored by PCR analysis as described above.

It is clear from the amplicon banding pattern in Fig 29, that X-inactivation in patient 4 was random. This can be seen by the equivalent intensities of the PCR bands after *HpaII* digestion of genomic DNA. It is also clear from these data that patient 4 had inherited two different copies of the X-chromosome. In studies not shown here, the two microsatellite repeat lengths were found to be the same as the repeat length sizes from his mother and it is likely that he has inherited both of his mother's X-chromosomes. Paternal DNA was not available to confirm this assumption.

Although we have shown random X-inactivation in white-cell DNA from our patient we have not ruled out skewing of X-inactivation as a feature in the aetiology of OTC deficiency as presenting females have been described where skewing is evident in the liver but not in circulating white-cells. This situation was reported, for example in a Japanese family of OTC carrier females (Yorifuji, T, et al, 1998), where X-inactivation patterns in the liver differed significantly from that of the peripheral blood leukocytes and even within the same liver. X-inactivation studies therefore, need to be carefully evaluated although in most cases, analysis of peripheral blood leukocytes satisfactorily reflects the X-inactivation status of the individual in other organs. Unfortunately in our study, a liver biopsy from patient 4 was not available to allow examination of this possibility.

The finding of normal coding and splice sequence for each of the 10 exons of the OTC gene from patient 4 was disappointing. However, this finding does not rule out an OTC gene defect as 20% of OTC deficient subjects present with similar results and are presumed to have a mutation elsewhere in the OTC gene, perhaps in the promoter region or in an intronic enhancer, or in some other gene implicated in OTC function.

To summarise the findings for patient 4. Initially, he started out as a strong candidate for the late-onset form OTC deficiency, but there is now considerable doubt about this diagnosis. This has arisen firstly, from the finding of a normal coding sequence for the OTC gene. However, 20 – 30% of patients with OTC deficiency do not have OTC gene mutations and are known as OTC "phenocopies". Our patient could thus possibly reside in this category into which we would put patient 2. However, the presence of Klinefelter's syndrome distinguishes this patient further and casts further doubt on the diagnosis as our patient shows heterodisomy for the X chromosome and must therefore show skewing of X-inactivation in his liver. This again is the less likely situation as <30% of carrier females show pathological skewing in the liver and then again, only a small portion of these woman show random skewing in white cells, which is the finding in our patient. (Yorifuji, T, et al, 1998)

Direct evidence for OTC deficiency in patient 4 would have come from enzyme studies on liver tissue but this was not an option for this laboratory. The evidence we have sought has been indirect and the findings now indicate that patient 4 is only a weak candidate for OTC deficiency but a much stronger candidate for that rare category of defect known as the OTC "Phenocopy".

Chapter 4.1: OTC microsatellite markers

Overview

This section details the findings of the prenatal diagnosis, using microsatellite markers, which was performed when the mother of patient 3 (kindred 3) became pregnant, and was found to be carrying a male foetus. At this stage although liver enzyme for OTC deficiency had not been performed, OTC deficiency was considered to be a strong enough candidate to justify the use of microsatellites in linkage analysis for the prenatal diagnosis. The reasons being, two previous male deaths in the neonatal period of this family, and the typical clinical presentation in patient 3, which included dramatically elevated blood ammonia and urinary orotic acid levels. Subsequent to the first prenatal diagnosis, OTC deficiency was conclusively shown by the mutation screening that was performed on DNA from patient 3. Consequently, the mother in this family is an obligate carrier for OTC deficiency.

Microsatellites

Microsatellites are DNA repeat sequences which have been used as markers in linkage studies and mapping, and occur frequently in the non-coding regions of the human genome. (URL 8) They consist of highly repetitive specific DNA sequences (DNA repeats) which occur as tandem repeats of varying length. They are also known as VNTR's (Variable Number Tandem Repeats), and are unique to each person. (URL. 9) The size of the repeating unit varies between 2 – 6bp, with CA repeats being the most commonly encountered. Typical examples of a di and tri-nucleotide repeat are shown in figure 30.

Fig 30. Examples of a dinucleotide (CA) and a trinucleotide (TAC) microsatellite repeat.

The flanking regions of each microsatellite are of unique sequence and are specific to each microsatellite. Individual microsatellites can thus be targeted by PCR using primer sequences selected from the flanking regions. The number of repeats varies from individual to individual and generally ranges between 10 to 28 in number. Following PCR the resulting amplicons can then sized and scored as alleles. Depending on the sizes of these alleles, an individual can be homozygous or heterozygous; homozygous, when the allele sizes are equal and heterozygous when the allele sizes are not the same. A given microsatellite locus can have as many as 5 to 15 alleles for a typical vertebrate population. It is possible to perform powerful genetic analysis when many or several loci are being analysed. Analyses of microsatellites are usually done by fluorescent labelling of PCR product and capillary electrophoresis (URL. 10). This study however, used amplicons labelled with radioactive [γ^{32}P] ATP, followed by chromatography on denaturing PAGE and autoradiography.

It has been estimated that microsatellites have an average mutation rate of 0-7x 10^{-3} mutations / locus / gamete / generation. (Brinkmann *et al*, 1998) Two favoured mechanisms are those involving unequal crossing over during meiosis

and strand-slippage during replication. (Forbes *et. al*, 1995). The latter mechanism appears to be the predominant one accounting for the variation in the lengths of microsatellites. The variation in length of the microsatellite is likely to occur during the lagging strand synthesis at the replication fork (Fig 31). During the dissociation of the polymerase complex (Fig 31c), a newly synthesized DNA strand slips, leading to the formation of a transient bulge. In the absence of DNA repair, the repeat increases in length. Alternatively, if the transient bulge forms on the template strand, the repeat may be shortened (not shown in Fig 30) (URL. 11).

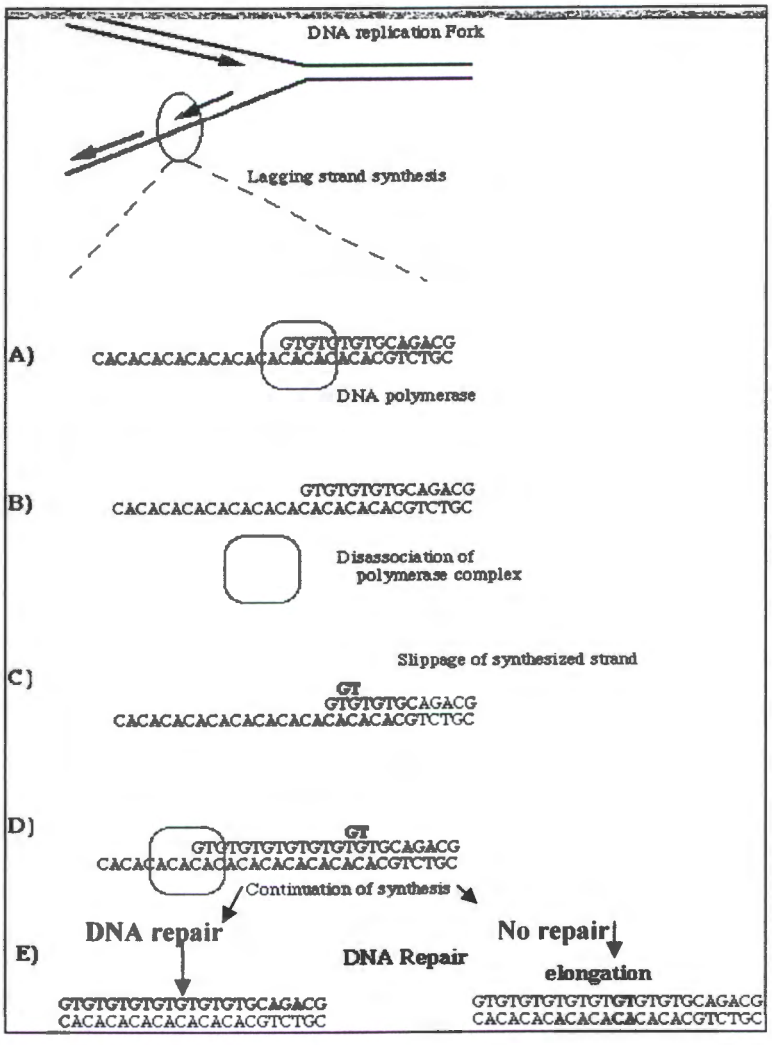


Fig 31. The Strand-slippage replication model of microsatellite length mutation (taken from URL 12).

Applications of satellites

Microsatellites have three useful features. Firstly, they are suitable for PCR amplification because of their small size (less than 100bp). Secondly, single copy nuclear DNA makes up the flanking regions, allowing for specificity in the PCR reaction and thirdly, they have high mutation rates. (URL. 12)

"Microsatellites have found extensive use in genome mapping studies and have also served as ideal markers for defective alleles in segregation analysis of familial disorders". (URL. 13) Other applications of microsatellites include population genetics, prenatal diagnosis, paternity testing and forensics analysis.

Chapter 4.2 Methods

4.2.1. Primer end-labelling for a sizing ladder or gene.

4.2.1.1. Labelling protocol

The reaction mix for a ladder of 10 µl volume was as follows:

10 x PNK buffer	1.0 µl
-40 Primer (5 µM)	0.8 µl
Distilled water	4.2 µl
[γ^{32}P] ATP	3.0 µl
*Diluted T4 PNK (5 U/µl)	1.0 µl
	10 µl

The reaction mix for a microsatellite of 20 µl volume was as follows:

10 x PNK buffer	2.0 µl
Primer Forward (20 pM)	5.0 µl
Distilled water	9.5 µl
[γ^{32}P] ATP	1.5 µl
*Diluted T4 PNK (5 U/µl)	2.0 µl
	20 µl

The samples were incubated at 37°C for 2 hours. Time is not critical and the tubes can be left overnight. Samples were heat inactivated at 90°C for 3 minutes or 65°C for 5 minutes, given a brief spin and stored at -20°C until use.

*(Dilute 30 U 1 in 6, i.e. 0.6 µl PNK + 3.0 µl PNK buffer. NB. The diluted enzyme is unstable)

4.2.2. Protocol for sizing ladder synthesis

4.2.2.1. PCR preparation of a DNA ladder using cycle sequencing

A PCR reaction mixture with a 35.6 µl volume was prepared as follows:

M13 DNA single stranded	5.0 µl
10 x Taq buffer	4.47 µl
Radiolabelled -40 Primer	2.83 µl
Distilled water	18.53 µl
Taq (5 U/µl)	0.3 µl
$MgCl_2$	4.47 µl
	35.6 µl

Four microlitres (4 µl) of ddATP + dNTP and ddTTP + dNTP mixes were aliquoted into two clean tubes (this mix contained the dNTP's and the specific ddNTP).

Aliquots of 500 µl were made up as follows:

Tube1 (ddATP+dNTP)	**tube 2 (ddTTP+dNTP)**
25 µl dNTP	25 µl dNTP
65 µl ddNTP	75 µl ddNTP
410 µl distilled water	400 µl distilled water
500 µl	500 µl

Into each ddATP and ddTTP tube, 16 µl of the above reaction mix was aliquoted and overlaid with mineral oil.

4.2.2.2. PCR Thermocycling

The following programme was used:

Step i. Denaturation at 94°C for2 minutes

This was set for 1 cycle

ii. denaturation at 94°C for 30 seconds

iii. annealing at 55°C for 30 seconds

iv. extension at 72°C for 1 minute

This was repeated for 28 cycles

v. Final extension at 72°C for 5-10 minutes

The reactions were terminated by the addition of 8 µl formamide stop mix. Both tubes were mixed together and stored at -20°C. The samples were denatured for 2-5 minutes @ 95°C before loading. Aliquots of the ladder (4 µl) were included in each run.

4.2.2.3. Preparation of sizing ladder using the Sequenase KIT

A reaction mix with a 29 µl volume was prepared as follows:

M13 DNA single stranded	10.0 µl
Reaction buffer	4.0 µl
Radiolabelled -40 Primer	10.0 µl
Distilled H_2O	5.0 µl
	29 µl

The sample was incubated at 65°C for 2 minutes, slowly allowed to cool to room temperature for 15-30 minutes, centrifuged and cooled on ice.

To the above tube the following was added:

Distilled water	7.0 µl
DTT (0.1M)	2.0 µl
*Diluted sequenase	1.0 µl
	10 µl

The 39 µl mix was divided equally (16 µl) into the 2 termination tubes.

The termination tubes had been prepared in advance, had been preheated to 37°C and now contained the following: **tube 1;** 4 µl ddATP; **tube 2,** 4 µl ddTTP. The two tubes were gently mixed and incubated at 37°C for 5 minutes followed by 70°C for 7 minutes and then 14 µl of stop solution was added to each reaction tube. The two mixes were combined, mixed, spun and stored at -20°C. The ladder was heated to 95°C for 5 minutes before loading onto the gel.

*(Dilute 0.5 µl sequenase, 0.5 µl pyrophosphate and 3 µl buffer)

4.2.3. Radioactive PCR reaction

4.2.3.1 PCR protocol

A reaction mix containing a 10.0µl volume was prepared as follows:

10 x Reaction Buffer	1.0 µl
50 mM $MgCl_2$	0.4 µl
5 mM dNTP's	0.66 µl
Reverse primer (20 pM)	0.33 µl
dH_20	6.2 µl
Taq Polymerase enzyme	0.066 µl
End-labelled Forward primer (20 pM)	1.33 µl
DNA (0.5 µg/µl)	0.5 µl
	10 µl

The 10.0 µl reaction sample was overlaid with 10 µl light mineral oil (Sigma) and briefly centrifuged. The tubes were placed in a thermocycler.

4.2.3.2. Thermocycling

An appropriate temperature profile for the thermocycling process was derived from the sequence of the forward and reverse primers.

The following programme was used:

Step i. Denaturation at 95°C for2 minutes

This was set for 1 cycle

ii. denaturation at 95°C for 45 seconds

vi. annealing at 55°C for 45 seconds

vii. extension at 72°C for 45 seconds

This was repeated for 30 cycles

viii. Final extension at 72°C for 5-10 minutes

This temperature profile was suitable for all primers used.

To each sample 4 µl of stop solution was added and the samples were stored until ready for use.

4.2.4. Polyacrylamide gel electrophoresis

4.2.4.1. Preparation of plates

The plates were prepared as in section 2.4.2 with a few slight modifications. No plate glue was used on either plate. Shark-tooth combs of size 1mm were used for the formation of wells.

The surfaces of both plates were thoroughly washed with detergent and 70% ethanol and allowed to dry completely. Gel slick was applied to one plate and vigorously rubbed onto the surface. The plates were assembled and secured with the aid of bulldog clips and with spacers (0.4 mm) separating the two plates. Shark-tooth combs of size 1mm were used for the formation of wells.

4.2.4.2. Preparation of PAGE gels

Gels were prepared from the following mix:

6% cold Polyacrylamide working stock	80.0 ml
APS (Ammonium persulfate)	280 µl
TEMED	64 µl

4.2.4.3. Sample preparation and electrophoresis

The gel was pre-electrophoresed for 30 minutes at 65W in 1 x TBE buffer. Prior to loading, PCR samples and the ladder were heated for 3 minutes @ 95°C and immediately placed on ice. Samples were loaded and the gel was run for 3 hours @ 65W.

4.2.4.4. Drying and autoradiography of the gel

At the end of electrophoresis, the power to the gel was disconnected and the TBE was discarded from the buffer chambers. The lower chamber was disposed in the radioactive waste container as it contained unincorporated radiolabel and radioactively-labelled short DNA molecules. A thin spatula was inserted between the plates and twisted to gently separate the plates. The gel adhered to the larger non-silanized plate. 3MM Chromatography paper was cut to a suitable size and in one smooth movement it was gently pressed down to ensure good gel to paper contact. Starting at one corner at the well end of the gel, the paper was peeled back and the gel adhered securely to it. A layer of Saran plastic wrap was placed on top of the gel and the paper/gel/plastic was then trimmed. The gel was then positioned, paper side down on gel drier and dried under vacuum @ 80°C for at least 90 minutes. In a darkroom illuminated with a safety light, the gel was placed in direct contact with a film sheet (autorad), inside a film cassette. The film was exposed for its desired length of time (12-24 hours) and at an appropriate temperature (room temperature). The time of exposure depended on the strength of the radioactivity in the sample, and in most cases, was determined empirically by making multiple exposures for different lengths of time.

4.2.5. Visualisation of the Microsatellites

4.2.5.1. Protocol for developing the X-ray film

After exposure, the film was removed from the cassette in the darkroom (using the safety light only) for developing. The film was immersed in the developer solution for 90 seconds, then in stop solution for 30 seconds and the in the fixer solution for 2 minutes. The film was then placed in running tap water for 5 minutes and left to dry. The alleles were then scored according to the relative positions of the exposed bands.

Chapter 4.3.1: Microsatellite Results

A search of the human genome database for microsatellites flanking the OTC gene revealed three that seemed suitable. These were DXS8090, DXS1068 and DXS8113, with the outside pair being separated by a distance of 3cM, which gives recombination probability of approximately 3%. The search was done using the following web address:
http://www.ncbi.nlm.nih.gov/genemap98/map.cgi?MAP=GB4&BIN=610&MARK=WI-9177

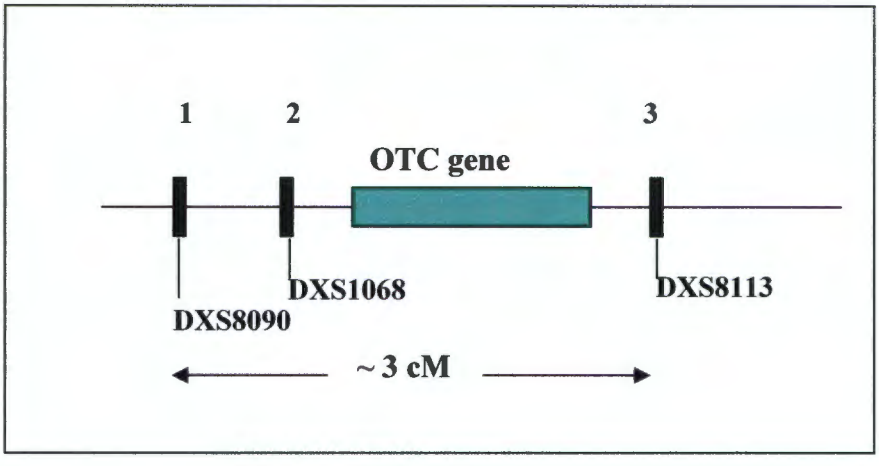


Figure 32. Diagram of the OTC gene showing the location of the microsatellite markers DXS8090, DXS1068, and DXS8113.

Table 2 Details of the selected microsatellite markers which flank the OTC gene.

Locus	**Heterozygosity index**	**PCR Primer Sequence 5' – 3'**	**Amplicon size range**
DXS 8090	0.81	Forward GGGTGAAATTCCATCACAAA Reverse ACAAATGCAGATGTACAAAAAATA	154 – 172
DXS 1068	0.79	Forward CCTCTAAAGCATAGGGTCCA Reverse CCATCTGAGAACACGCTG	245 - 259
DXS 8113	0.68	Forward CCTCTACATAGGCACATGC Reverse CCAAAGGAGTTATTTGTCACCT	229 – 239

DNA from the mother, unaffected son, affected son (patient 3), previously affected son and the foetus was amplified by PCR in the presence of ^{32}PαdATP and primer pairs for each of the 3 microsatellites. PCR products were separated on a 6% polyacrylamide gel.

For microsatellites to be informative in this family, it would be necessary for the mother of patient 3 to carry different length repeats on her X-chromosomes. This would enable the mutant allele to be identified through PCR of the OTC allele from the previously affected sibling. Each of the microsatellites, was amplified from maternal DNA with only DXS8090 distinguishing between the two alleles by giving two bands on electrophoresis of PCR products (fig 33).

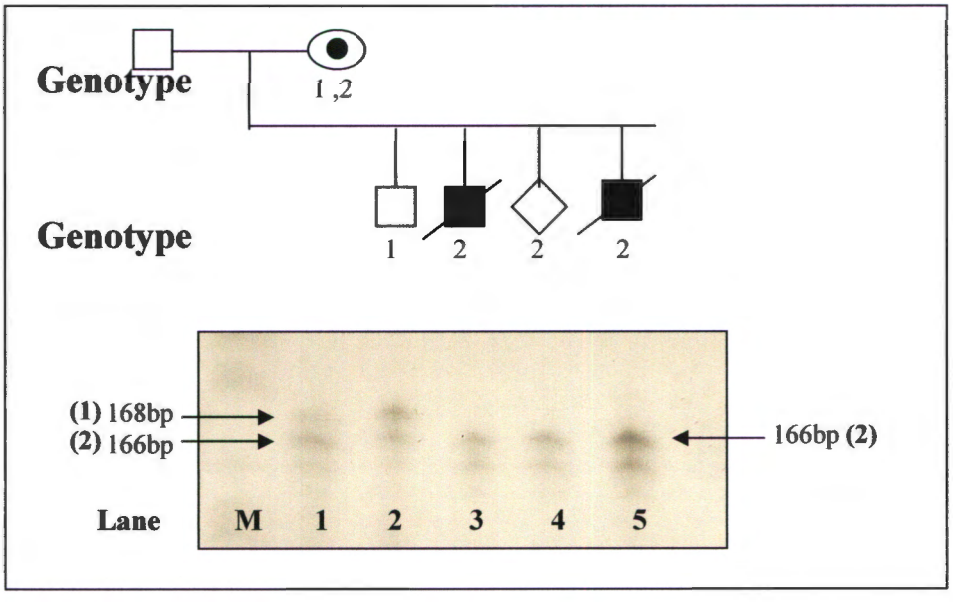


Figure 33. Autoradiograph showing OTC microsatellite marker DXS8090 for the family of patient 3. M depicts the sizing ladders used. Lane 1- mother (1,2), lane 2- unaffected son (1), lane 3- patient 3 (previously affected son) (2), lane 4- foetus (2) and lane 5- affected son (2). Allele 1 = 168bp and allele 2 = 166bp.

The two maternal alleles were clearly distinguishable, enabling accurate scoring for each sibling. It was clear from the autoradiograph that maternal allele 2 carried the mutant OTC gene as this was the allele inherited by both of the affected sons. Unfortunately this allele had also been inherited by the foetus and was thus indicative of OTC deficiency. The diagnosis of an affected male foetus was communicated to the parents who then elected to terminate the pregnancy. Approximately eighteen months later, the mother returned to the hospital, pregnant and requested another prenatal diagnosis. At the time of doing the microsatellite analysis the gender of the foetus was unknown. Even though the mutation was known at this stage, microsatellite analysis was used in the second prenatal because the screening of the general population was being undertaken at the time.

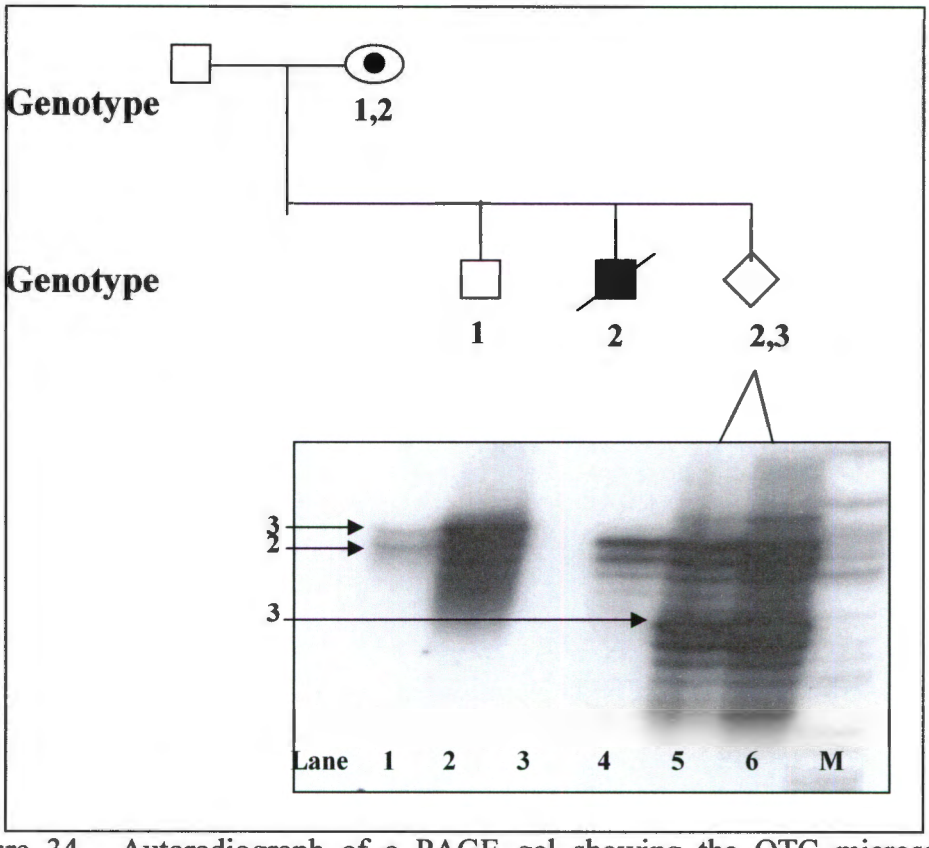


Figure 34. Autoradiograph of a PAGE gel showing the OTC microsatellite marker DXS8090 for the family of patient 3 (second pregnancy). Lane 1- mother, lane 2- unaffected son, lane 4- patient 3 (affected son), lane 5 & 6- foetus and M depicts the sizing ladder used. Allele 1 = 168bp and allele 2 = 166bp.

The DXS8090 genotype recorded for the foetus clearly demonstrated that the gender was female and that the defective OTC allele (allele 2) had been inherited from the mother, according carrier status to the foetus. The diagnosis of a carrier female foetus was communicated to the parents, who were eventually delivered of a healthy daughter.

Chapter 4.3.2: OTC Microsatellite markers in the South African Population
A survey of 3 ethnic groups

Heterozygosity indexes of DXS8090, DXS1068 and DXS8113 in the South African population.

The lack of informativeness in two out of the three microsatellites analysed in this family was unexpected, as both have yielded high heterozygosity indexes (<0.68) in other populations.

The primary objective of this study was to determine the range of repeat lengths and the heterozygosity indexes of DXS8090, DXS1068 and DXS8113 in South Africans from different racial backgrounds. These data would allow for a ranking of the microsatellites in order of increasing likelihood of being informative in future OTC diagnostic interventions. Unrelated individuals of the racial groupings African, Mixed ancestry and Caucasian, were chosen for this study.

DXS8090

The autoradiograph demonstrating the various allele sizes for microsatellite DXS8090 in the African population is shown in fig. 35 A and B. Males and females have been run on different gels.

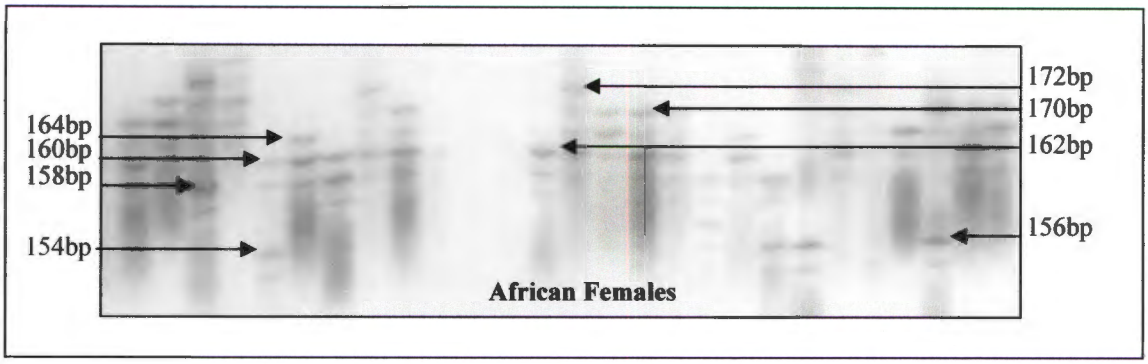


Figure 35 A. Autoradiograph of PAGE gels run to determine size distribution of the microsatellite marker DXS8090 in African females. The sizes shown represent the length in bp of the PCR amplicons; individual CA repeat numbers were not determined. The DNA ladder used to determine the size is not shown.

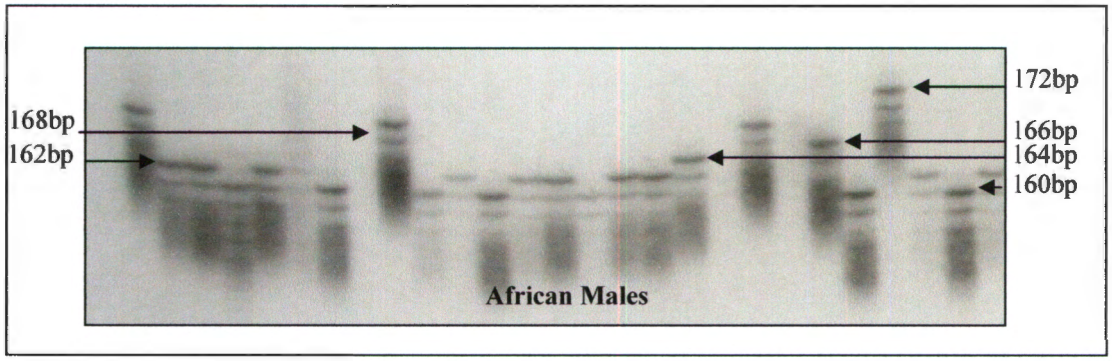


Figure 35 B. Autoradiograph of PAGE gels run to determine the size distribution of the microsatellite marker DXS8090 in African males. The DNA ladder used to determine the size is not shown.

The allele sizes, distribution and frequencies for this locus for the different ethnic groups are shown in Table 2 and fig. 36.

Table 3. Allele sizes distribution and frequencies for Microsatellite DXS8090

Allele sizes, distribution and frequencies for Microsatellite marker DXS8090						
	n		**n**		**n**	
Size	**Caucasian**	**Frequency**	**Mixed ancestry**	**Frequency**	**African**	**Frequency**
150	0	0	1	0.01	0	0
152	0	0	4	0.039	0	0
154	0	0	0	0	4	0.054
156	1	0.012	0	0	7	0.095
158	7	0.085	3	0.029	11	0.149
160	6	0.073	9	0.088	7	0.095
162	12	0.146	17	0.167	17	0.23
164	10	0.122	11	0.108	8	0.108
166	5	0.061	8	0.078	14	0.189
168	35	0.427	22	0.216	2	0.027
170	5	0.061	19	0.186	2	0.027
172	1	0.012	8	0.078	2	0.027

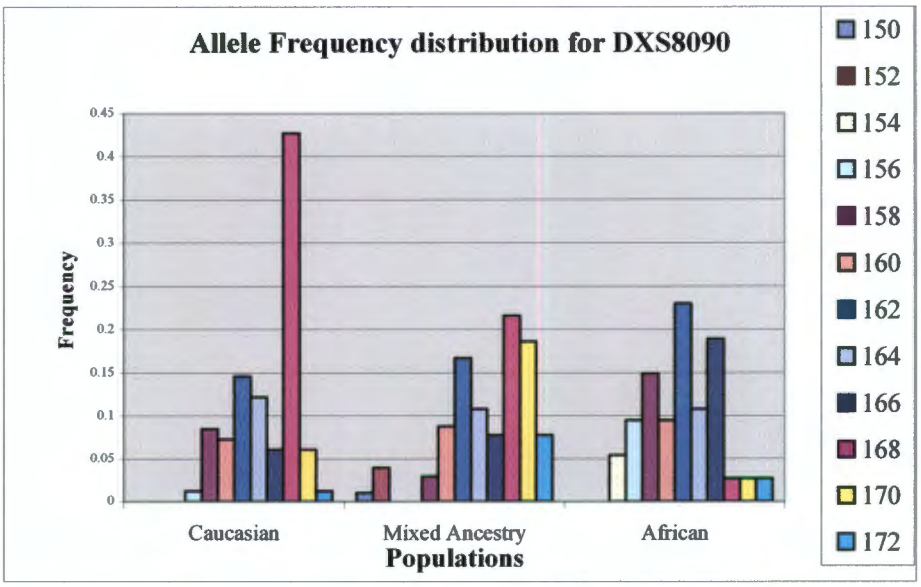


Figure 36. Graphical presentation of the allele frequency distribution amongst the three populations for microsatellite marker DXS8090.

DXS1068

The autoradiographs demonstrating the various allele sizes for microsatellite DXS1068 in the African and Caucasian populations is shown in fig 37 A and B.

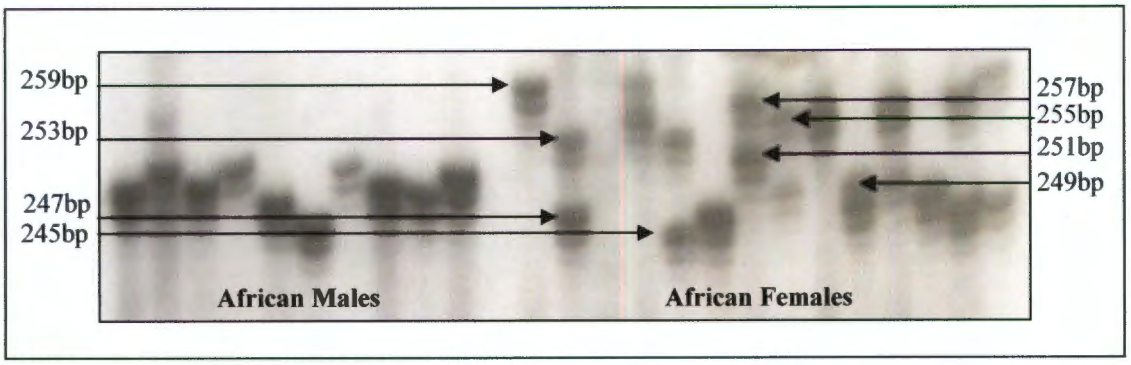


Figure 37 A. Autoradiograph of PAGE gels run to determine size distribution of the microsatellite marker DXS1068 in African males and females. The DNA ladder used to determine the size is not shown.

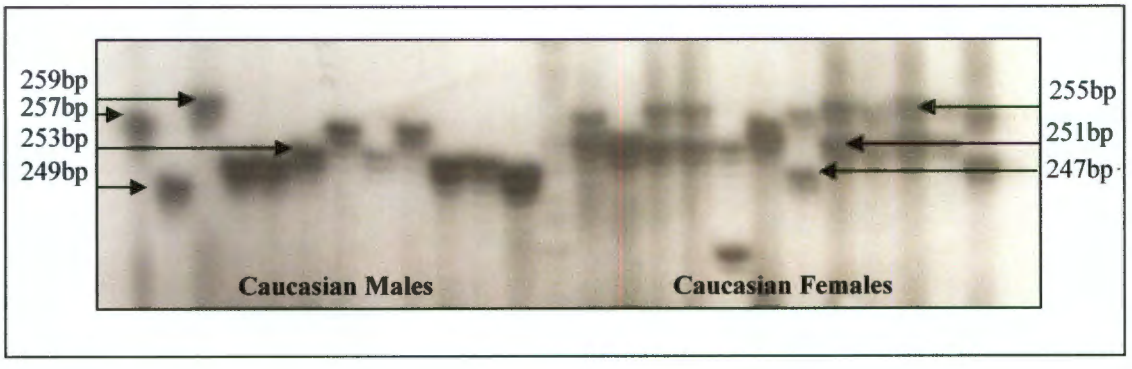


Figure 37 B. Autoradiograph of PAGE gels run to determine size distribution of the microsatellite marker DXS1068 in Caucasian males and females. The DNA ladder used to determine the size is not shown.

The allele sizes, distribution and frequencies for this locus for the different ethnic groups are shown in Table 3 and fig. 38.

Table 4. Allele sizes, distribution and frequencies for Microsatellite DXS1068

Allele sizes, distribution and frequencies for Microsatellite marker DXS1068						
	n		**n**		**n**	
Size	**Caucasian**	**Frequency**	**Mixed ancestry**	**Frequency**	**African**	**Frequency**
245	1	0.013	10	0.109	7	0.1
247	1	0.013	7	0.076	12	0.171
249	7	0.092	16	0.174	14	0.2
251	4	0.053	11	0.12	14	0.2
253	8	0.105	10	0.109	0	0
255	22	0.289	5	0.054	2	0.029
257	13	0.171	27	0.293	14	0.2
259	20	0.263	6	0.065	7	0.1

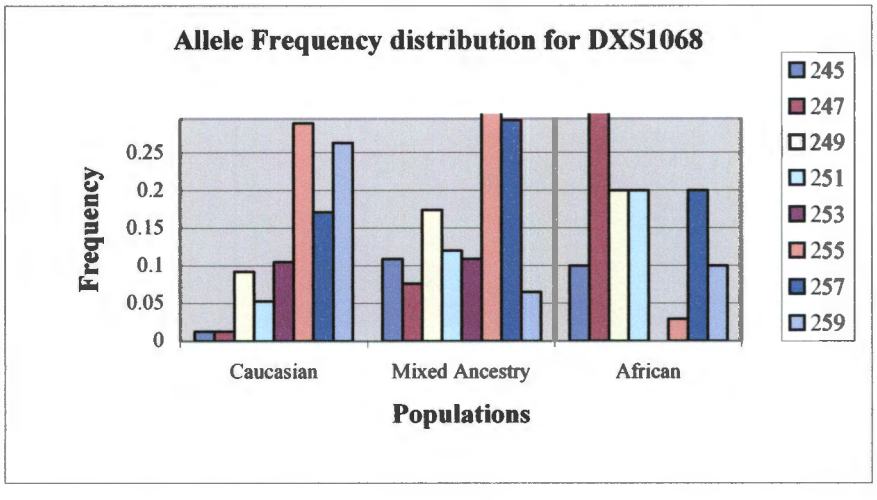


Figure 38. Graphical presentation of the allele frequency distribution amongst the three populations for microsatellite marker DXS1068.

DXS8113

The autoradiographs demonstrating the various allele sizes for microsatellite DXS8113 in the African population is shown in fig 39 A and B.

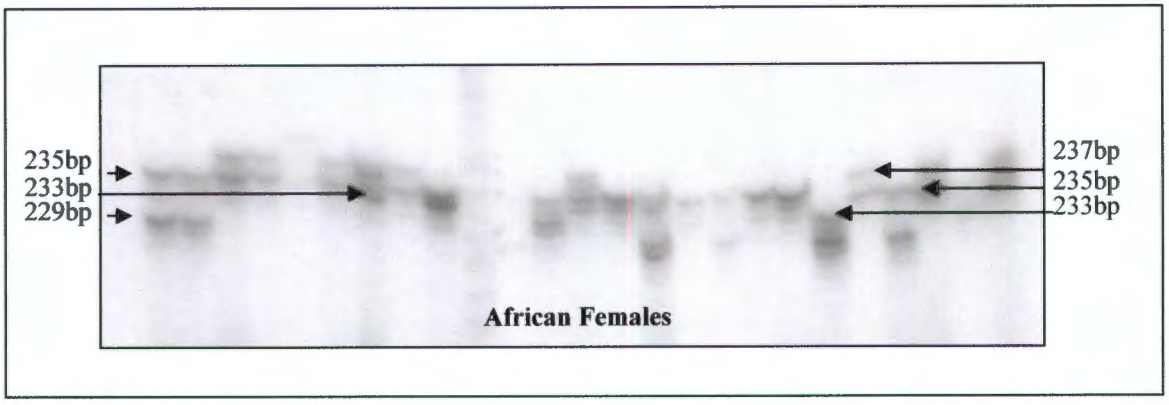


Figure 39 A. Autoradiography of PAGE gels run to determine the size distribution of the microsatellite marker DXS8113 in African Females. The DNA ladder used to determine the size is not shown.

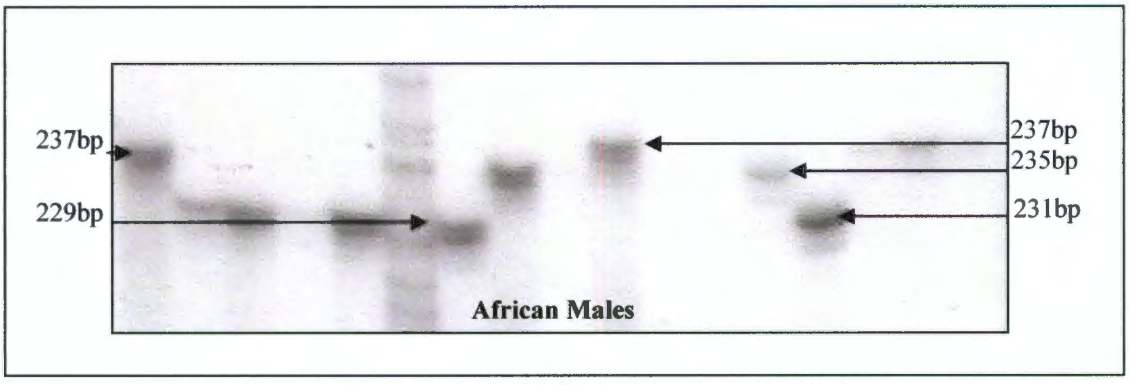


Figure 39 B. Autoradiography of PAGE gels run to determine the size distribution of the microsatellite marker DXS8113 in African Males. The DNA ladder used to determine the size is not shown.

The allele sizes and frequencies for this locus for the different ethnic groups are shown in Table 4 and fig. 40.

Table 5. Allele sizes, distribution and frequencies for locus DXS8113

Allele sizes, distribution and frequencies for Microsatellite marker DXS8113						
	n		**n**		**n**	
Size	**Caucasian**	**Frequency**	**Mixed ancestry**	**Frequency**	**African**	**Frequency**
227	0	0	1	0.012	0	0
229	7	0.08	11	0.128	10	0.132
231	13	0.148	18	0.209	3	0.039
233	12	0.136	0	0	6	0.079
235	15	0.17	33	0.384	40	0.526
237	35	0.398	20	0.233	16	0.211
239	6	0.068	3	0.035	1	0.013

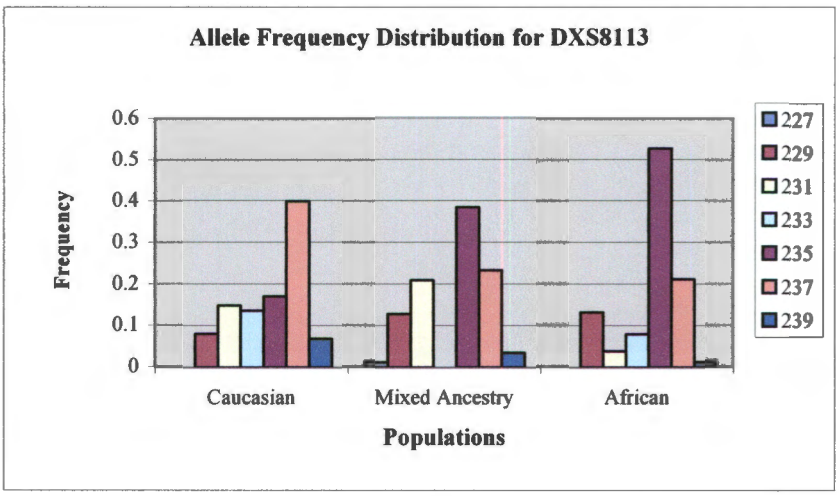


Figure 40. Graphical presentation of the allele frequency distribution amongst the three populations for microsatellite marker DXS8113.

A summary of the overall allele sizes and frequencies for the 3 microsatellites are represented in table 5.

Table 6. Summary of overall allele sizes and frequencies for the combined ethnic groups

Summary of overall allele sizes and frequencies for the combined ethnic groups								
DXS8090			**DXS1068**			**DXS8113**		
Size	**# alleles**	**Freq**	**Size**	**# alleles**	**Freq**	**Size**	**# alleles**	**Freq**
150	1	0.004	245	18	0.076	227	1	0.004
152	4	0.016	247	20	0.084	229	28	0.112
154	4	0.016	249	37	0.155	231	34	0.136
156	8	0.031	251	29	0.122	233	18	0.072
158	21	0.081	253	18	0.076	235	88	0.352
160	22	0.085	255	29	0.122	237	71	0.284
162	46	0.178	257	54	0.227	239	10	0.04
164	29	0.112	259	33	0.139			
166	27	0.105						
168	59	0.229						
170	26	0.101						
172	11	0.043						

Microsatellite Heterozygosity Indices

Microsatellite heterozygosity indices are a good predictor of the likelihood of a microsatellite marker being informative in family linkage studies such as prenatal diagnosis. The higher the index, the higher the probability of informativeness. These indices are tabulated in table 6. When calculating heterozygosity indices, the following formulas were used:

$$\textbf{Observed Heterozygosity} = \frac{\textbf{Heterozygotes}}{\textbf{Heterozygotes + Homozygotes}}$$

Expected Heterozygosity = 1 – Sum (frequency of each allele squared)

Table 7. Observed and expected heterozygosity indexes of each Microsatellite

	DXS8090		DXS1068		DXS8113	
	Observed	Expected	Observed	Expected	Observed	Expected
Caucasian	0.75	0.783	0.833	0.795	0.737	0.761
Mixed ancestry	0.85	0.857	0.75	0.833	0.778	0.737
African	0.875	0.854	0.833	0.83	0.875	0.653

4.4. Discussion and Conclusion

The impetus to look at microsatellites around the OTC gene stemmed from two requests from the parents of patient 3 for prenatal diagnosis in recent pregnancies. OTC deficiency cannot be diagnosed by measuring OTC activity from cultured amniocytes or aminiotic fluid, and the only recourse in this family was thus, DNA linkage analysis using a X-chromosome genotypes as defined by those inherited by the affected and unaffected siblings (Ch 2, fig 10). The OTC gene mutation in this family was unknown at this stage and it was therefore necessary to look at the X-chromosome genotypes of closely associated and flanking microsatellites markers.

Three microsatellites were selected for analysis and all gave readable gels after PCR and PAGE. Only one however, proved to be informative in this kindred in spite of high reported heterozygosity indexes. This microsatellite was successfully used to detect an affected male foetus in the first prenatal and a carrier female foetus in the second pregnancy. PCR of OTC exonic regions can now be used as an alternative approach should the parents again request pre-natal diagnosis, as the mutation in this family has subsequently been shown to be a large intragenic deletion, removing exons 5-10 (Ch 2, fig 21); diagnosis of an affected male foetus would thus be made on the inability to amplify product from these exons.

The lack of informativeness for two of these microsatellites in kindred 3 was unexpected as heterozygosity indexes reported in the literature are all >0.68 However, these indexes are from populations of European ancestry, and while they are likely to apply universally, it was deemed reasonable to determine their values in Africans and Mixed ancestry from South Africa and to examine their length diversities. These data would also allow for their ranking and thus order of selection in future prenatal testing.

Heterozygosity indexes were determined in a study cohort, which consisted of 91 females, 27 of whom were African, 40 mixed ancestry and 24 Caucasian. Both the observed and expected indexes have been given in the results section, and while there was general agreement between the indexes, markers DXS8113 and DXS1068 differed in the African and Mixed ancestry groups. While this may be indicative of some deviation from Hardy-Weinberg equilibrium, the small sample size examined would suggest that this is due to sampling error. For the purposes of microsatellite ranking it is better to work with the expected indexes as they take the length variations into account.

The expected heterozygosity indexes observed for the three microsatellite markers were all in the high range and above 0.65, varying from 0.65 – 0.88. There is thus little reason to choose between these markers on the basis of their indexes, except perhaps in Africans where DXS8113 gave an index of 0.65. More important in microsatellite selection however, is the ease of amplification and the ability to score individual bands following PAGE and autoradiography for ^{32}P labelled PCR products. In this context, DXS8090 would the marker of choice as it gave the clearest bands (Fig 35A) and also gave the widest range of repeat lengths.

In conclusion, the microsatellite markers used in this study have been found to be useful in the indirect analysis of the OTC gene and have allowed for the detection of an affected foetus in the first prenatal and a carrier foetus in the second. Ideally OTC deficiency in the first foetus should have been confirmed through a liver biopsy on the abortus, but logistical difficulties precluded this follow-up procedure.

REQUEST FOR DNA TESTING

DNA Diagnostic Laboratory
Dept. Chemical Pathology
ICH Building,
Rondebosch, 7700

Tel: (021) 6585223 Fax: (021) 6891287

Blood volumes : Children 2-3ml in and EDTA tube
Adults 5-10 ml in an EDTA tube

Each tube should be inverted to mix and should be clearly labelled with the patient's name and DOB
Keep blood in fridge at 4°C until able to send to laboratory

Please DO NOT send specimens on ice or frozen.

Please fill in all the information requested:

Surname:_____ First Name(s):_____

New Family: Yes ☐ No ☐ (If no, please fill in family name) Family name: _____

Medical Aid: _____ Medical Aid No: _____

Sex: M ☐ F ☐ Date of Birth: Day:_____ Month:_____ Year:
_____

Number of children: _____

Ethnic Origin : (please indicate ancestry of both your mother and father
_____

Contact Address: _____ Town: _____ Fax:
Tel: _____

Referring Doctor/Sister: _____ Town: _____ Fax:
Tel: _____

Hospital or Address: _____ Town: _____ Fax:
Tel: _____

Reason for Referral (Clinical diagnosis):.....
.....

Testing required: ☐ Galactosemia ☐ Spinal Muscular Atrophy ☐ Cystic Fibrosis (ΔF508)

☐ Lipoprotein Lipase ☐ McArdles ☐ MCAD ☐
Other.....

Pedigree Drawing

CONSENT FOR DNA ANALYSIS AND STORAGE

1 I, _____ , request that an attempt be made using genetic material to assess the probability that: I / my child / my unborn child (*Delete where not applicable*) might have inherited a disease-causing mutation in the gene for:_____

2 I understand that the genetic material for analysis is to be obtained from: blood cells/skin sample/other (specify) (*Delete where not applicable*) :

3 I request that **no** portion of the sample be stored for later use. ☐ (*Mark if applicable*)
Or
I request that a portion of the sample be stored indefinitely for (*Delete where not applicable*):
(a) possible re-analysis
(b) analysis for the benefit of members of my immediate family
(c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

4. The results of the analysis carried out on this sample of stored biological material will be made known to me, via my doctor, in accordance with the relevant protocol, if and when available.
In addition, I authorise that they may be made known to: (*Delete where not applicable*) :
- other doctors involved in my care
- the following family members: _____
- other: _____

5 I authorise/ do not authorise my doctor(s) (delete where applicable) to provide relevant clinical details to the genetic Clinic, Red Cross Children's Hospital

6. I have been informed that:
(a) there are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.
(b) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
(c) the genetics laboratory is under an obligation to respect medical confidentiality .
(d) genetic analysis may not be informative for some families or family members.
(e) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
(f) where biological material is used for research purposes, there may be no direct benefit to me.

7. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.

8. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:**

_____ DATE: _____

Patient signature _____ **Witnessed consent** _____

For Laboratory use only:

DNA number: _____ *Vol.Blood:* _____ *(ml)* *Other:* _____

Date Received: Year: *Month:* *Day:* *Computer Index No:*

Appendix

Mutational Analysis

Table 8. List of Primers for the 10 OTC Exons. (5' – 3')

OTCExon	Forward Primer	Reverse Primer
1	agttttcaagggcatagaatc	gttttatgcatcaccatgattcc
2	accatagtacatgggtcttttctg	ggactggtagttaactggaac
3	cacttatttgggggctagttatta	gtcttcaccttcaatccctct
4	gagatgatggccaattctttg	tcagattctgaaatcagctttgg
5	ggcattatttaagcataattatctt	cacttaagcaagtcaggaatta
6	ttcatctccttcatcccgtg	gtgatttaagagaggggttagtttc
7	aaataagatttaaattccttcctc	aggcaataatagctttacat
8	actgtcccatgaagttattta	atttgttttcttctggccttca
9	atagtcaaaaagtggtcttatccc	atctcacttgcttattatttccc
10	aggtccctaagcagactgtcg	tcattctgttactgaagaacattgc

The lyophilised primers were reconstituted in TE Buffer at stock concentrations of 500pmoles/µl and stored at –70 °C. Working solutions were diluted to 20pmoles/µl concentrations and stored at –20 °C.

Table 9. $MgCl_2$ Concentration and Temperature Profile.

Exon	$MgCl_2$ Concentration	Annealing Temperature
1	1.5mM	55°C
2	1.5mM	55°C
3	1.0mM	51°C
4	1.5mM	55°C
5	1.0mM	51°C
6	1.5mM	55°C
7	1.0mM	51°C
8	1.5mM	51°C
9	1.5mM	55°C
10	1.5mM	58°C

Ethidium Bromide

1000X Stock Solution (0.5mg/ml)

50mg Ethidium Bromide in 100ml ddH_2O

Working Ethidium Bromide (0.5ug/ml)

Dilute stock solution 1 in 1000 (for gels)

0.5M EDTA

Dissolve 186.1g $Na_2EDTA.2H_2O$ in 400ml ddH_2O, adjust pH to 8.0 with 10M NaOH (use NaOH pellets if necessary). Make up to 1 litre with dH_2O.

50X TAE Stock

242g Tris base

57.1ml Glacial acetic acid

100ml 0.5M EDTA

Make up to 1 litre with dH_2O and Autoclave.

1X Working TAE

Dilute 50X stock TAE 1 in 50 with dH_2O (10ml 50X TAE in 500ml dH_2O).

10X TBE Electrophoresis Buffer

108g Tris base

55g Boric acid

40ml 0.5M EDTA

Make up to 1 litre with dH_2O.

1X Working TBE

Dilute 10X Stock – 100ml in 1 litre dH_2O.

TE Buffer

10mM TrisHCl pH 7.6

1mM EDTA pH 8.0 (disodium salt)

1ml 1M Tris pH 7.6 and 200 µl 0.5M EDTA pH 8.0 were dissolved in 100ml dH_2O.

DNA Extraction Protocols

EDTA Blood Extraction Method

Low salt buffer (TKM1)

10mM Tris-HCL pH7.6

10mM KCL

10mM $MgCl_2$

2mM EDTA (disodium salt)

0.121g Tris, 0.075g KCl, 0.203g $MgCl_2$ and 0.075g EDTA were dissovled in 100ml ddH_2O with pH adjusted to 7.6 with HCl.

Nonidet P-40 (Igepal)

Supplied by Sigma, catalogue number I 3021

High salt buffer (TKM2)

10mM Tris-HCL pH7.6

10mM KCL

10mM $MgCl_2$

0.4M NaCl

2mM EDTA (disodium salt)

0.121g Tris, 0.075g KCl, 0.203g $MgCl_2$, 2.338g NaCl and 0.075g EDTA were dissovled in 100ml ddH_2O with pH adjusted to 7.6 with HCl.

10% Sodium Dodecyl Sulphate (SDS)

10g SDS were dissolved in 100ml ddH_2O.

6M NaCl

35.06g NaCl in 100ml ddH_2O (Note that this is a saturated solution).

100% Ethanol

70% Ethanol

70ml absolute ethanol and 30ml distilled water.

DNA Extraction from Heparinized blood

Heparin (Sigma 1000 units/ml)

Cell lysis buffer pH 8.0

0.32M Sucrose

5mM $MgCl_2$

10mM TrisHCl pH 7.6

1% Triton X-100

109.5g Sucrose, 5ml of 1M $MgCl_2$ and 10ml of 1M TrisHCl was dissolved in 990ml of distilled water, autoclaved and while the buffer was warm, 10ml Triton X-100 was added and the mix was stored at 4^0C.

1% Triton X-100

109.5g Sucrose, 5ml of 1M MgCl2 and 10ml Tris-HCl were dissolved in 990ml ddH_2O, autoclaved and while the buffer was warm, add 10ml Triton X-100 and store at 4 °C.

Wash buffer

10mM NaCl

10mM EDTA (disodium salt) pH 8.0

0.58g NaCl and 3.72g EDTA were dissolved in 1000ml ddH_2O and stored at 4 °C.

20% Sarkosyl Solution

20g N-Lauroylsarkosine (sodium salt)(Sigma) were dissolved in 10ml ddH_2O, aliquoted and stored at –20 °C.

7.5M Ammonium Acetate

57.81g Ammonium acetate were dissolved in 100ml ddH_2O, aliquted and stored at –20 °C.

6.0M Guanidinium Chloride

57.32g Guanidinium chloride were dissolved in 100ml ddH_2O and stored at 4 °C.

DNA Extraction from cell cultures

Saline

0.9g NaCl in 100ml ddH_2O.

100uM EDTA

3.72mg EDTA (MW=372.24) in 100ml ddH_2O.

4M NaCl

33.37g NaCl in 100ml ddH_2O.

Saline/EDTA

50ml 100uM EDTA and 3.7ml 4M NaCl in 200ml ddH_2O.

10% SDS

10g SDS in 100ml ddH_2O.

10mg/ml Proteinase K

100mg Proteinase K (from *Tritirachium album*) (Rouche) was dissolved in 10ml distilled water, aliquoted and stored at 4^0C.

Proteinase K

20mg Proteinase K/ml (Boeringer Mannheim #1413783).

6M NaCl

35.1g NaCl in 100ml ddH_2O.

Isopronanol

Supplied by Merck, Catalogue number AB009634

70% ethanol

70ml absolute ethanol and 30ml dH_2O

Polymerase Chain Reaction

Materials and Reagents:

Thermal Cycler

0.5ml thin walled sterile microcentrifuge tubes.

Deoxynucleotidetriphosphates (dNTP's):

The following lyophilised Boehringer Mannheim products were used:

dATP disodium salt	Cat No. 103985
dCTP disodium salt	Cat No. 104043
dGTP disodium salt	Cat No. 104108
dTTP trisodium salt	Cat No. 104272

Stock solutions were made in ultra pure water, the pH adjusted to 7.0 with 50mM Tris base made in ultra pure water and sterile filtered. The 10mM solutions were made from the stock solutions and 20ul of each 10mM dNTP stock solution were mixed to give a 2mM dNTP working solution. Stocks and working solutions were stored at –20 °C.

$MgCl_2$ (Sigma)

A 1.00M $MgCl_2$ was diluted to 25mM in dH_2O.

10 x PCR buffer

The Promega stock solution was used.

Taq DNA polymerase

The Promega Taq was used (10U/ul).

Primer/DNA dilution buffer

10mM Tris pH 7.6

1mM EDTA

1ml 1M Tris pH 7.6 and 200 µl 0.5M EDTA pH 8.0 were dissolved in 100ml distilled water. The solution was filtered, aliquoted and stored at -20^{0}C.

DNA template

Working stocks (0.1µg/µl) of the isolated DNA was made using DNA dilution buffer.

Forward and Reverse Primers (See table1)

All primers were diluted in Primer dilution buffer. Stock solutions of 500pM was made of each primer and stored at -75^{0}C. Working stocks of 20pM was used in the PCR reactions and stored @ -20^{0}C.

6 x DNA loading buffer

0.25% Bromophenol blue

30% Glycerol

0.25g Bromophenol blue and 30ml glycerol was dissolved in a total volume of 100ml distilled water and stored at 4^{0}C.

SSCP loading dye

10mM NaOH

95% Formamide

0.05% Bromophenol blue

0.05% Xylene Cyanol

9.5ml Formamide, 10µl 10M NaOH, 250µl 2% Bromophenol blue and 250 µl 2% Xylene cyanol were all combined.

Stock plate glue solution

50µl γ-Methacryloxypropyl trimethoxysilane (Sigma) added to 100ml absolute ethanol.

Siliconizing agent

Dimethyl dichlorosilane solution (BDH), Catalogue number D3879

Ultra pure urea

Supplied by Sigma, Catalogue number U0631

40% Acrylamide stock (39.6: 0.4)

39.6g Acrylamide (BDH Laboratory Supplies) and 0.4g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on a magnetic stirrer in 100ml of dH_2O.

30.8% Acrylamide stock (30 : 0.8)

30g Acrylamide (BDH Laboratory Supplies) and 0.8g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on the magnetic stirrer in 100ml of dH_2O.

10% APS (Sigma)

0.1g Ammonium Persulphate (APS) dissolved in 1ml dH_2O.

N,N,N',N'-Tetramethylethylenediamine (TEMED) (Promega)

Ammoniacal silver solution

0.1% $AgNO_3$

0.1% NaOH

0.1% NH_4OH

2g NaOH was dissolved in just less than 2 litres of distilled water, then 8ml of NH_4OH was added and covered while stirring gently to avoid evaporation of the NH_3

Immediately before use 2g $AgNO_3$ was added while stirring.

Developing solution

0.005% Citric acid

0.02% Formalin (34% stock)

0.1g of the citric acid and 1.176ml of the formalin was dissolved in 2 litres of dH_2O.

Solution1

2g $AgNO_3$ was dissolved in 2 litres of dH_2O.

Solution2

30g of NaOH, 0.2g NaBH4 and 8ml formaldehyde was dissolved in 2 litres of dH_2O.

Solution 3

15g Na_2CO_3 was dissolved into 2 litres of dH_2O.

Sequence Analysis

40% Sucrose (w/v)

10g of sucrose was dissolved in 25ml of dH_2O, filtered, aliquoted and stored at -20^0C.

1.0% Agarose gel

1.0g (w/v) low melting point agarose (Whitehead Scientific)

100 ml 1 x TAE

2.5µl Ethidium Bromide

30% Acrylamide (29:1)

29g of Acrylamide and 1g of NN'-methylenebis-acrylamide dissolved on the magnetic stirrer in 100ml of dH_2O.

RFLP and Southern Blot Analysis

Restriction Enzymes

Bam HI, Hind III, and Pvu II, supplied by Boerhinger and Mannheim.

Incubation Buffers for Restriction Enzymes

0.25M HCl

21.48 ml HCl was made up to a total volume of 1000ml ddH_2O

0.4M NaOH

16g NaOH were dissolved in 1000ml ddH_2O

Nylon Membrane

Nylon Hybond N+ membrane (Amersham)

Whatman No. 3 Filter Paper

Supplied by Merck, Catalogue number 234/0310/21

Hybridization Buffer

0.26M Na_2HPO_4

1.0mM EDTA

0.24M SDS

BSA

18.6g Na_2HPO_4, 1.0ml EDTA(500mM), 35.0g SDS, and 5.0g BSA were dissolved in a total volume of 500ml with ddH_2O.

20X SSC Solution

175.3g NaCl and 88.2g trisodium citrate were dissolved in a total volume of 1000ml with the pH adjusted to 7.0 with 10N NaOH and autoclaved.

10% Sodium Dodecyl Sulfhate

10g SDS were dissolved in 100ml ddH_2O.

Wash Solutions:

2XSSC/0.1%SDS

Mix 200ml 20XSSC and 20ml 10%SDS in a total volume of 2000ml with ddH_2O.

0.2XSSC/0.1%SDS

Mix 20ml 20XSSC and 20ml 10%SDS in a total volume of 2000ml with ddH_2O.

Hyperfilm ^{TM} MP High Performance Autoradiography Film

Supplied by Amersham Life Science

X-inactivation

10 x Buffer L

Supplied by Promega in a 1.5ml volume

Hpa II enzyme

Supplied by Promega with a concentration of 10U/µl in a 20ul volume

Casein

0.0076g Casein (Sigma) diluted in 7.6ml 10mM Tris pH 8.0 and had a concentration of 1mg/ml. The reagent was then aliquoted and stored @ -20^0C.

Bovine Serum Albumin (BSA)

Supplied by Promega to a final concentration of 0.1mg/ml

Androgen primer forward (ANDR-f)

Manufactured by Whitehead Scientific. Primers were diluted with primer dilution buffer to a final working concentration of 20pM/µl and stored at -20^0C.

5' TGC GCG AAG TGA TCC AGA ACC-3'

Androgen primer reverse (ANDR-r)

Manufactured by Whitehead Scientific. Primers were diluted with primer dilution buffer to a final working concentration of 20pM/µl and stored at -20^0C.

5' TGG GCT TGG GGA GAA CCA TCC -3'

DNA template

Working stocks (0.5µg/µl) of the isolated DNA was made using DNA dilution buffer.

20% Acrylamide (19:1)

19g Acrylamide (BDH Laboratory Supplies) and 1.0g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on the magnetic stirrer in 100ml of distilled water.

Microsatellites

10 x PNK buffer

Supplied by the USB Corporation.

-40 Primer

Boehringer Mannheim manufactured the primer with a stock concentration of 5µM.

T4 PNK

The enzyme was supplied by the USB Corporation and had a stock concentration of 30U/µl. A working stock of T4 PNK was used at a concentration of 5U/µl.

[γ^{32}P] ATP

The radioactive [γ^{32}P] ATP stock was 150mCi/ml > 5000Ci/mmol and was supplied by Amersham PB15068.

M13 DNA single stranded

Supplied by USB Corporation with a stock concentration of 0.2µg/µl

Sequenase KIT

Supplied by Boehringer Mannheim. A 1:2 dilution of the sequenase was made before with pyrophosphate and briefly spun.

40% Acrylamide stock

250.0g Acrylamide (BDH Laboratory Supplies) and 13.1g NN'-methylenebis-acrylamide (BDH Laboratory Supplies) dissolved on a magnetic stirrer in 657ml of warm dH_2O, stored in a brown bottle @ 4^0C.

6% Polyacrylamide working solution

A volume of 15.0ml 40% Polyacrylamide stock solution, 42.0g Urea, 10.0ml 10 x TBE and 33ml of water were mixed to give a final volume of 100ml of the working solution. This mix could only be stored for 2 weeks @ 4^0C.

10 x TBE

108g Tris base, 55.0g Boric acid and 40ml of 0.5M EDTA (pH 8.0) dissolved in 1000ml of dH_2O.

Developer solution

Stop solution

2% acetic acid

Fixer solution

A 1:3 dilution was made and the pH and silver content was checked.

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